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(54) Title: DNA EXPRESSION SYSTEMS BASED ON ALPHAVIRUSES

(57) Abstract

Efficient protein production from cloned DNA in animal cells has been hampered by the lack of suitable expression systems. The requirements of such an expression system are (1) to produce functional or immunogenic forms of protein molecules in a wide variety of animal cells, (2) high efficiency and (3) technical simplicity. The present invention is related to a technical solution to this problem. A DNA molecule encoding protein sequences is inserted into engineered variants of the cDNA of a positive stranded RNA virus genome from alphavirus which then, via RNA transcription and transfection into tissue culture cells, is used to produce either chimaeric virus particles for immunization or recombinant virus for protein production. Because of optimized conditions of transfection and the nature of the virus replication the present system combines both simplicity and safety in terms of handling, efficiency in terms of level of protein and RNA production, as well as broad host range.

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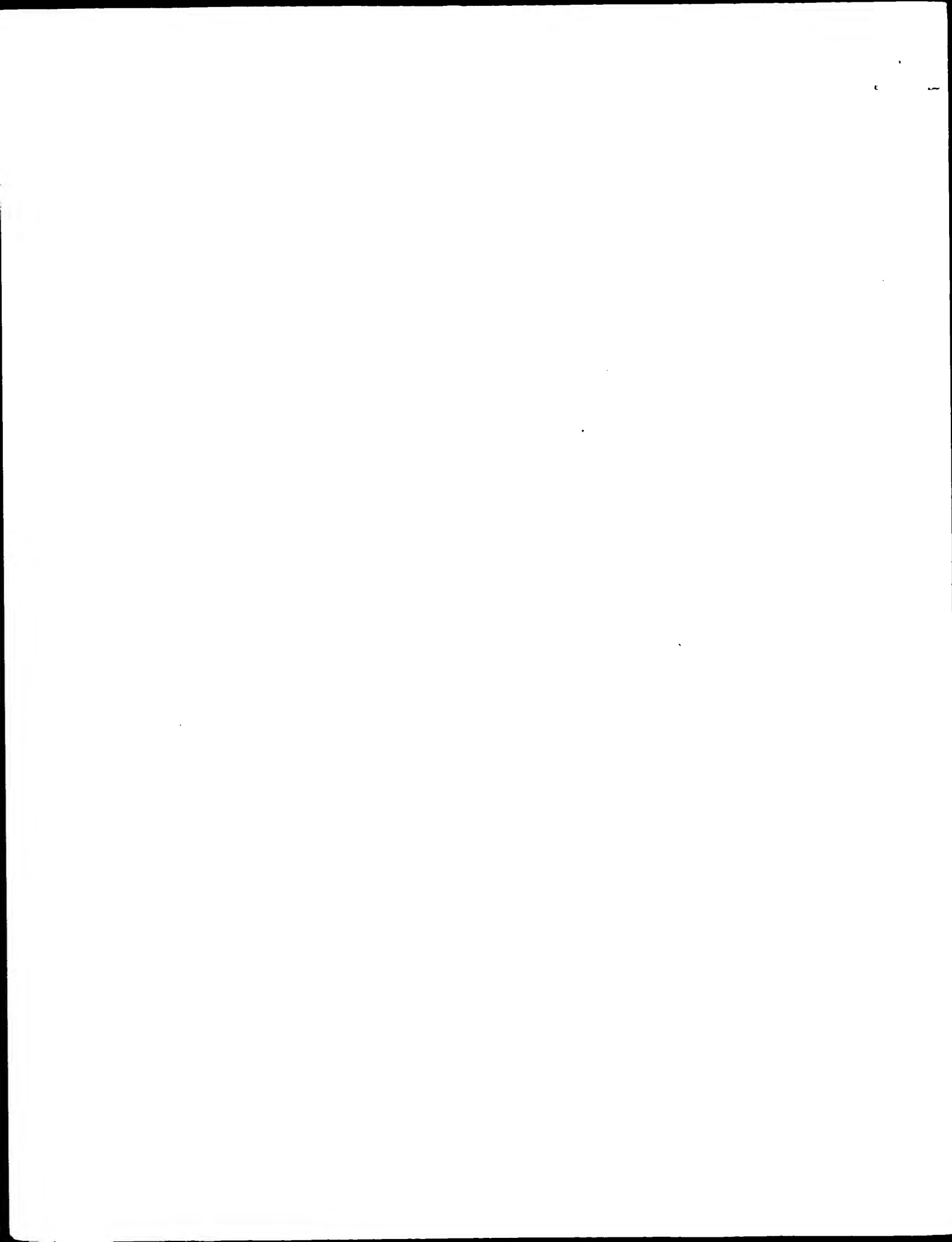
DNA EXPRESSION SYSTEMS BASED ON ALPHAVIRUSES

The present invention is related to DNA expression systems based on alphaviruses, which systems can be used to transform animal cells for use in the production of desired products, such as proteins and vaccines, in high yields.

The rapid development of biotechnology is to a large extent due to the introduction of recombinant DNA technique, which has revolutionized cellbiological and medical research by opening new approaches to elucidate the molecular mechanisms of the cell. With the aid of the techniques of cDNA cloning, large numbers of interesting protein molecules are characterized each year. Therefore, a lot of research activity is today directed to elucidate the relationship between structure and function of these molecules. Eventually this knowledge will increase our possibilities to preserve healthiness and combat diseases in both humans and animals. Indeed, there is today a growing list of new "cloned" protein products that are already used as pharmaceuticals or diagnostics.

In the recombinant DNA approaches to study biological questions, DNA expression systems are crucial elements. Thus, efficient DNA expression systems, which are simple and safe to use, give high yields of the desired product and can be used in a variety of host cells, especially also in mammalian cells, are in great demand.

Many attempts have been made to develop DNA expression systems, which fulfill these requirements. Often, viruses have been used as a source of such systems. However, up to date none of the existing viral expression systems fulfill all these requirements in a satisfying way. For instance, the <u>Baculovirus</u> expression system for cDNA is extremely efficient but can be used only in insect cells (see Reference 1 of the list of cited references; for the sake of convenience, in the following the cited references are only identified by the number they have on said list). As many important molecules will have to be produced and processed in

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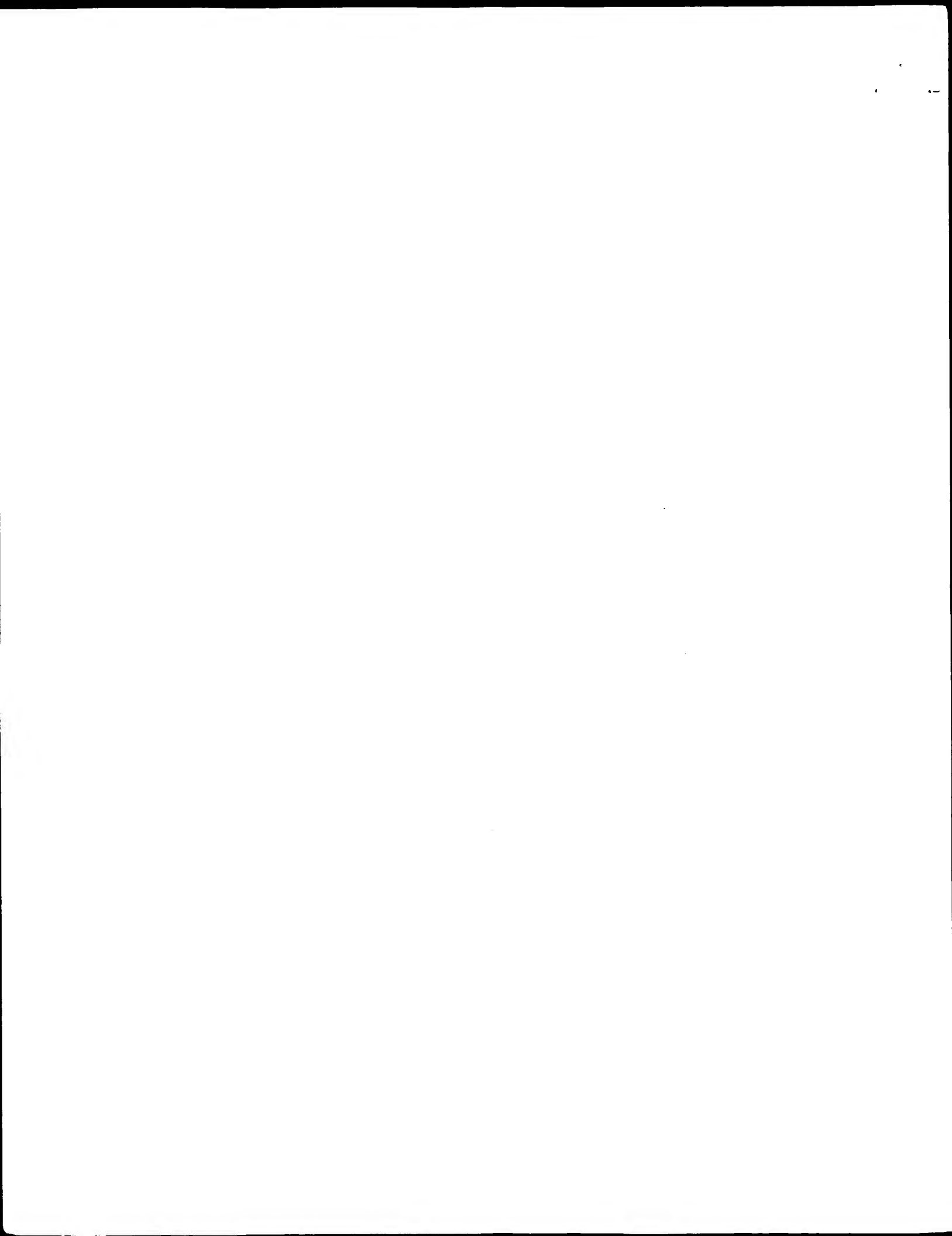
cells of mammalian origin in order for them to become
active, this system cannot be used in such cases.
Furthermore, the Baculovirus cDNA expression system is
not practically convenient for analysis of the
relationship between structure and function of a pro-
tein because this involves in general the analysis of
whole series of mutant variants. Today it takes about
6-8 weeks to construct a single Baculo recombinant
virus for phenotype analyses. This latter problem is
also true for the rather efficient Vaccinia recombinant
virus and other contemporary recombinant virus cDNA
expression systems (2,3). The procedure to establish
stably transformed cell lines is also a very laborious
procedure, and in addition, often combined with very
low levels of protein expression.

Hitherto, most attempts to develop viral DNA expres-
sion systems have been based on viruses having DNA
genomes or retroviruses, the replicative intermediate
of the latter being double stranded DNA.

Recently, however, also viruses comprising RNA
genomes have been used to develop DNA expression
systems.

In EP 0 194 809 RNA transformation vectors derived
from (+) strand RNA viruses are disclosed which
comprise capped viral RNA that has been modified by
insertion of exogenous RNA into a region non-essential
for replication of said virus RNA genome. These vectors
are used for expression of the function of said exo-
genous RNA in cells transformed therewith. The RNA can
be used in solution or packaged into capsids. Further-
more, this RNA can be used to generate new cells having
new functions, i.e. protein expression. The invention
of said reference is generally claimed as regards host
cells, (+) strand RNA viruses and the like. Neverthe-
less, it is obvious from the experimental support
provided therein that only plant cells have been trans-
formed and in addition only Bromo Mosaic virus, a plant

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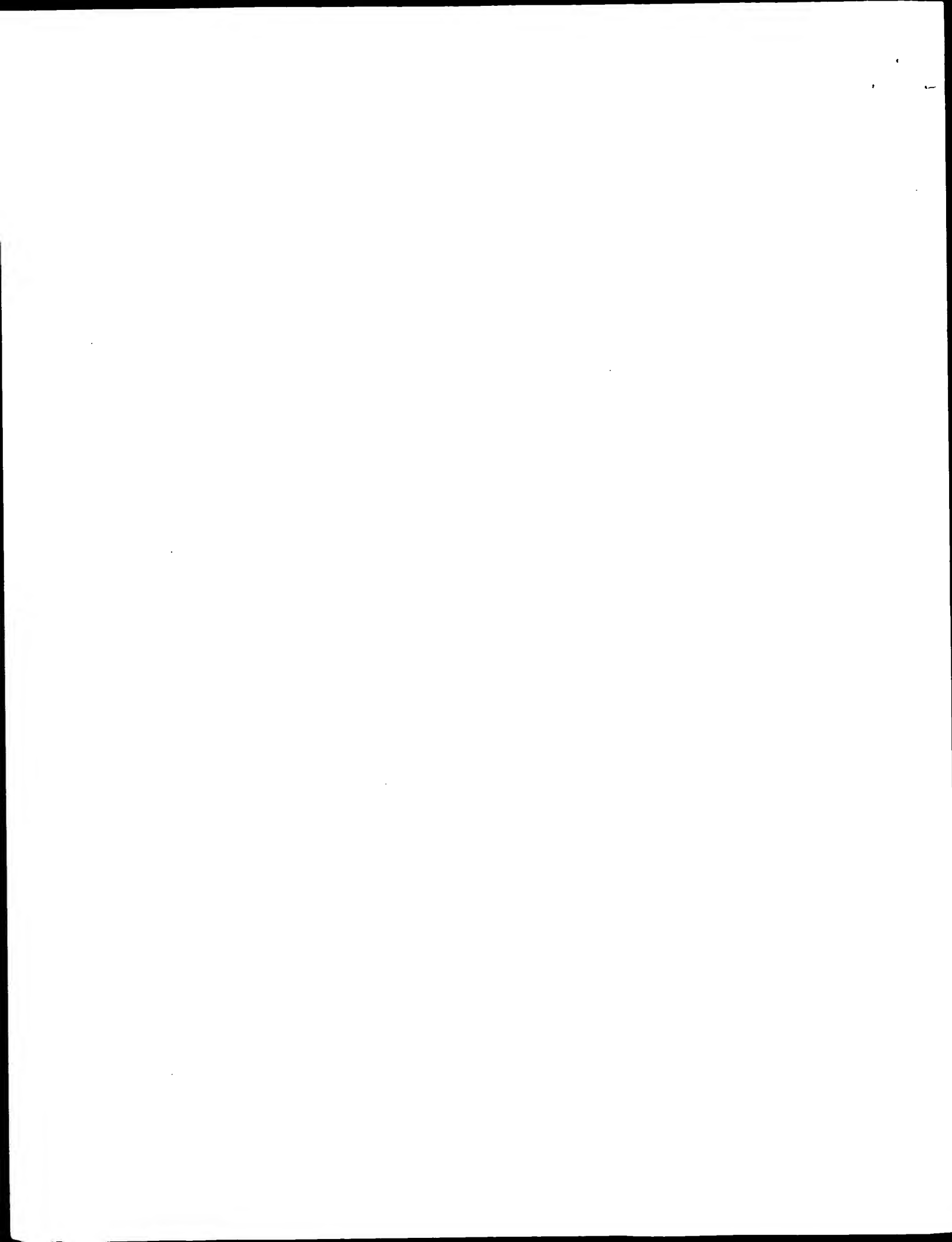
virus, has been used as transformation vector.

Although it is stated in said reference that it is readily apparent to those skilled in the art to convert any RNA virus-cell system to a useful expression system for exogenous DNA using principals described in the reference, this has not been proven to be true in at least the case of animal cell RNA viruses. The reasons for this seem to be several. These include:

1)   Inefficiencies in transfecting animal cells with in vitro transcribed RNA;

2)   Inefficiency of apparently replication competent RNA transcripts to start RNA replication after commonly used transfection procedures;

3)   The inability to produce high titre stocks of recombinant virus that does not contain any helper virus;

4)   The inability to establish stable traits of transformed cells expressing the function of the exogenous RNA.

In Proc. Natl. Acad. Sci. USA, Vol 84, 1987, pp 4811-4815 a gene expression system based on a member of the Alphavirus genus, viz. Sindbis virus, is disclosed which is used to express the bacterial CAT (chloramphenicol acetyltransferase) gene in avian cells, such as chicken embryo fibroblasts.

Xiong et al., Science, Vol 243, 1989, 1188-1191 also disclose a gene expression system based on Sindbis virus. This system is said to be efficient in a broad range of animal cells. Expression of the bacterial CAT gene in insect, avian and mammalian cells inclusive of human cells is disclosed therein.

Even though it is known from prior art that one

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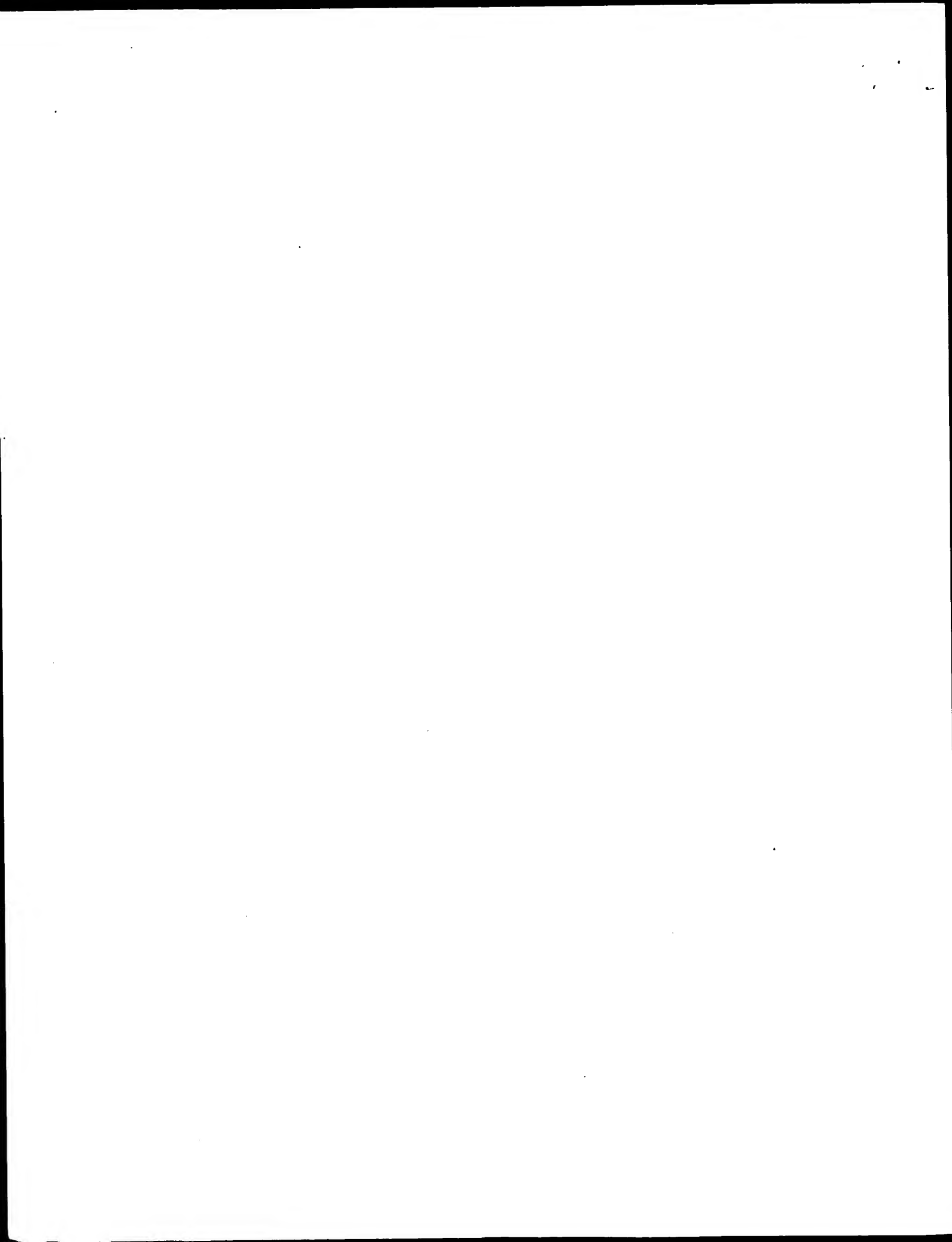
member of the Alphavirus genus, the Sindbis virus, can
tolerate insertion and direct the expression of at
least one foreign gene, the bacterial chloramfenicol
acetyl transferase (CAT) gene, it is evident from the

5   results described that both systems described above are
both ineffective in terms of exogenous gene expression
and also very cumbersome to use. Hence, neither system
has found any usage in the field of DNA expression in
animal cells today.

10   In the first example a cDNA copy of a defective
interfering (DI) virus variant of Sindbis virus was
used to carry the CAT gene. RNA was transcribed in
vitro and used to transfect avian cells and some CAT
protein production could be demonstrated after in-

15   fecting cells with wild-type Sindbis virus. The latter
virus provided the viral replicase for expression of
the CAT construct. The inefficiency of this system
depends on 1) low level of initial DI-CAT RNA transfec-
tion (0.05-0.5 % of cells) and 2) inefficient usage of

20   the DI-CAT RNA for protein translation because of
unnatural and suboptimal protein intitation translation
signals. This same system also results in packaging of
some of the recombinant DI-CAT genomes into virus
particles. However, this occurs simultaneously with a

25   very large excess of wild-type Sindbis virus produc-
tion. Therefore, the usage of this mixed virus stock
for CAT expression will be much hampered by the fact
that most of the replication and translation activity
of the cells infected with such a stock will deal with

30   the wild-type and not with recombinant gene expression.
Much of the same problems are inherent to the other
Sindbis expression system described. In this an RNA
replication competent Sindbis DNA vector is used to
carry the CAT gene. RNA produced in vitro is shown to

35   replicate in animal cells and CAT activity is found.
However, as only a very low number of cells are trans-
fected the overall CAT production remains low. Another

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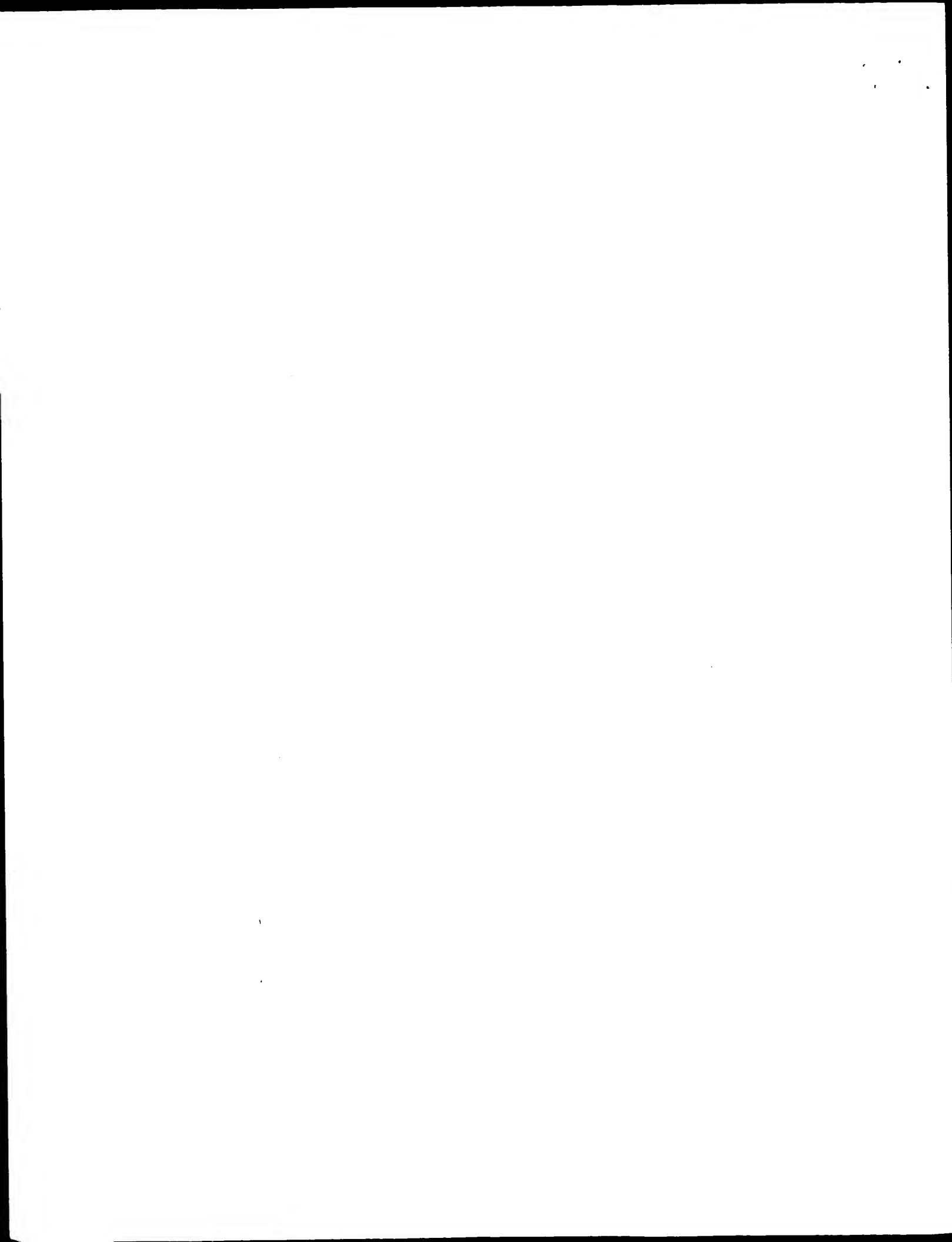
possible explanation for this is that the Sindbis con-
struct used is not optimal for replication. Wild-type
Sindbis virus can be used to rescue the recombinant
genome into particles together with an excess of wild-
type genomes and this mixed stock can then be used to
express a CAT protein via infection. However, this
stock has the same problems as described above for the
recombinant DI system. The latter paper shows also that
if virus is amplified by several passages increased
titres of the recombinant virus particles can be
obtained. However, one should remember that the titre
of the wild-type virus will increase correspondingly
and the original problem of mostly wild-type virus
production remains. There are also several potential
problems when using several passages to produce a mixed
virus stock. As there is no selected pressure for pre-
servation of the recombinant genomes these might easily
1) undergo rearrangements and 2) become outnumbered by
wild-type genomes as a consequence of less efficient
replication and/or packaging properties.

Another important aspect of viral DNA expression
vectors is use thereof to express antigens of unrelated
pathogens and thus they can be used as vaccines against
such pathogens.

Development of safe and effective vaccines against
viral diseases has proven to be quite a difficult task.
Although many existing vaccines have helped to combat
the worldwide spread of many infectious diseases, there
is still a large number of infectious agents against
which effective vaccines are missing. The current pro-
cedures of preparing vaccines present several problems:
(1) it is often difficult to prepare sufficiently large
amounts of antigenic material; (2) In many cases there
is the additional hazard that the vaccine preparation
is not killed or sufficiently attenuated; (3) Effective
vaccines are often hard to produce since there is a
major difficulty in presenting the antigenic epitope in

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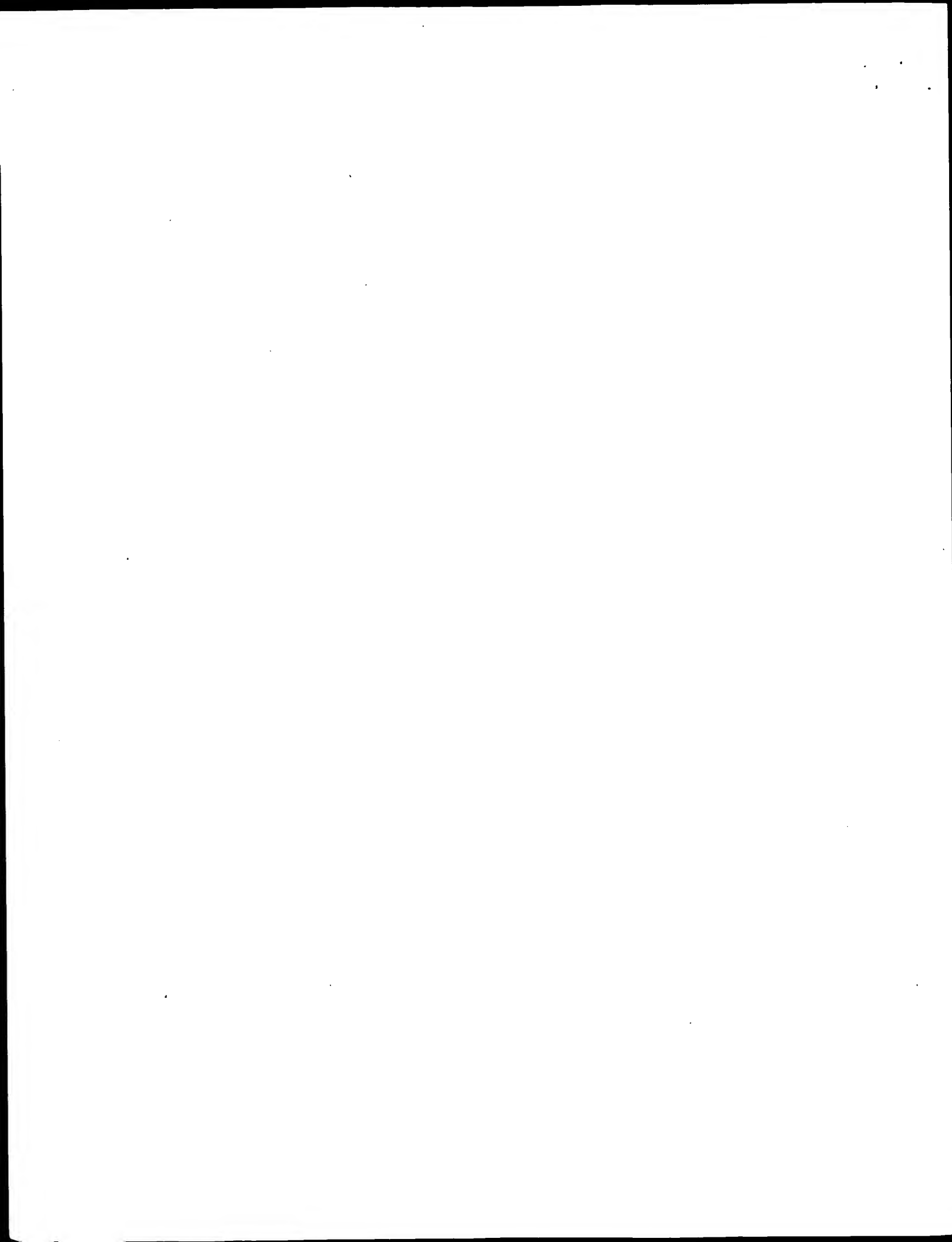
an immunologically active form; (4) In the case of many viruses, genetic variations in the antigenic components results in the evolution of new strains with new serological specificities, which again creates a need for the development of new vaccines.

Two types of viral DNA vectors have been developed in order to overcome many of these problems in vaccine production. These either provide recombinant viruses or provide chimaeric viruses. The recombinant viruses contain a wild-type virus package around a recombinant genome. These particles can be used to infect cells which then produce the antigenic protein from the recombinant genome. The chimaeric viruses also contain a recombinant genome but this specifies the production of an antigen, usually as part of a normal virus structural protein, which then will be packaged in progeny particles and e.g. exposed on the surface of the viral spike proteins. The major advantages of these kind of virus preparations for the purpose of being used as a vaccine are 1) that they can be produced in large scale and 2) that they provide antigen in a natural form to the immunological system of the organism. Cells, which have been infected with recombinant viruses, will synthesize the exogenous antigen product, process it into peptides that then present them to T cells in the normal way. In the case of the chimaeric virus there is, in addition, an exposition of the antigen in the context of the subunits of the virus particle itself. Therefore, the chimaeric virus is also called an epitope carrier.

The major difficulty with these kind of vaccine preparations are, how to ensure a safe and limited replication of the particles in the host without side effects. So far, some success has been obtained with vaccinia virus as an example of the recombinant virus approach (69) and of polio virus as an example of a chimaeric particle (70-72). As both virus variants are

based on commonly used vaccine strains one might argue
that they could be useful vaccine candidates also as
recombinant respectively chimaeric particles (69-72).
However, both virus vaccines are combined with the risk

5   for side effects, even severe ones, and in addition
these virus strains have already been used as vaccines
in large parts of the population in many countries.

As is clear from the afore mentioned discussion there
is much need to develop improved DNA expression systems

10   both for an easy production of important proteins or
polypeptides in high yields in various kinds of animal
cells and for the production of recombinant viruses or
chimaeric viruses to be used as safe and efficient
vaccines against various pathogens.

15   Thus, an object of the present invention is to
provide an improved DNA expression system based on
virus vectors which can be used both to produce
proteins and polypeptides and as recombinant virus or
chimaeric virus, which system offers many advantages

20   over prior art.

To that end, according to the present invention there
is provided an RNA molecule derived from an alphavirus
RNA genome and capable of efficient infection of animal
host cells, which RNA molecule comprises the complete

25   alphavirus RNA genome regions, which are essential to
replication of the said alphavirus RNA, and further
comprises an exogenous RNA sequence capable of expres-
sing its function in said host cell, said exogenous RNA
sequence being inserted into a region of the RNA mole-

30   cule which is non-essential to replication thereof.

Alphavirus is a genus belonging to the family
Togaviridae having single stranded RNA genomes of
positive polarity enclosed in a nucleocapsid surrounded
by an evelope containing viral spike proteins.

35   The Alphavirus genus comprises among others the
Sindbis virus, the Semliki Forest virus (SFV) and the
Ross River virus, which are all closely related.

According to a preferred embodiment of the invention, the Semliki Forest virus (SFV) is used as the basis of the DNA expression system.

The exogenous RNA sequence encodes a desired genetic trait, which is to be conferred on the virus or the host cell, and said sequence is usually complementary to a DNA or cDNA sequence encoding said genetic trait. Said DNA sequence may be comprised of an isolated natural gene, such as a bacterial or mammalian gene, or may constitute a synthetic DNA sequence coding for the desired genetic trait i.e. expression of a desired product, such as an enzyme, hormone, etc. or expression of a peptide sequence defining an exogenous antigenic epitope or determinant.

If the exogenous RNA sequence codes for a product, such as a protein or polypeptide, it is inserted into the viral RNA genome replacing deleted structural pro-tein encoding region(s) thereof, whereas a viral epi-tope encoding RNA sequence may be inserted into structural protein encoding regions of the viral RNA genome, which essentially do not comprise deletions or only have a few nucleosides deleted.

The RNA molecule can be used per se, e.g. in solution to transform animal cells by conventional transfection, e.g. the DEAE-Dextran method or the calcium phosphate precipitation method. However, the rate of transforma-tion of cells, and, thus the expression rate can be expected to increase substantially if the cells are transformed by infection with infectious viral par-ticles. Thus, a suitable embodiment of the invention is related to an RNA virus expression vector comprising the RNA molecule of this invention packaged into infec-tious particles comprising the said RNA within the alphavirus nucleocapsid and surrounded by the membrane including the alphavirus spike proteins.

The RNA molecule of the present invention can be packaged into such particles without restraints pro-

vided that it has a total size corresponding to the
wild type alphavirus RNA genome or deviating therefrom
to an extent compatible with package of the said RNA
into the said infectious particles.

These infectious particles, which include recombinant
genomes packaged to produce a pure, high titre recombi-
nant virus stock, provides a means for exogenous genes
or DNA sequences to be expressed by normal virus par-
ticle infection, which as regards transformation
degree, is much more efficient than RNA transfection.

According to a suitable embodiment of the invention
such infectious particles are produced by cotrans-
fection of animal host cells with the present RNA which
lacks part of or the complete region(s) encoding the
structural viral proteins together with a helper RNA
molecule transcribed in vitro from a helper DNA vector
comprising the SP6 promoter region, those 5' and 3'
regions of the alphavirus cDNA which encode cis acting
signals needed for RNA replication and the region
encoding the viral structural proteins but lacking
essentially all of the nonstructural virus proteins
encoding regions including sequenses encoding RNA
signals for packaging of RNA into nucleocapsid par-
ticles, and culturing the host cells.

According to another aspect of the invention effi-
cient introduction of the present RNA into animal host
cells can be achieved by electroporation. For example,
in the case of Baby Hamster Kidney (BHK) cells a trans-
formation degree of almost 100 % has been obtained for
the introduction of an RNA transcript derived from SFV
cDNA of the present invention. This makes it possible
to reach so·high levels of exogenous protein production
in every cell that the proteins can be followed in
total cell lysates without the need of prior concentra-
tion by antibody precipitation.

By electroporation, it is also possible to obtain a
high degree of cotransfection in the above process for

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production of infectious particles comprising packaged
RNA of the present invention. Essentially all animal
cells will contain both the present RNA molecule and
the helper RNA molecule, which leads to a very effi-
cient trans complementation and formation of infectious
partcles. A pure recombinant virus stock, consisting of
up to 10^9-10^{10} infectious particles, can be obtained
from 5×10^6 cotransfected cells after only a 24 h
incubation. Furthermore, the so obtained virus stock is
very safe to use, since it is comprised of viruses
containing only the desired recombinant genome, which
can infect host cells but can not produce new progeny
virus.

Theoretically, a regeneration of a wild-type virus
genome could take place when producing the recombinant
virus in the contransfected cells. However, the possi-
bility to avoid spread of such virus can be eliminated
by incorporating a conditionally lethal mutation into
the structural part of the helper genome. Such a muta-
tion is described in the experimental part of this
application. Thus, the virus produced with such a
helper will be noninfectious if not treated in vitro
under special conditions.

The technique of electroporation is well known within
the field of biotechnology and optimal conditions can
be established by the man skilled in the art. For
instance, a BioRad Gene pulser apparatus (BioRad,
Richmond, CA, USA) can be used to perform said process.

The RNA molecule of the present invention is derived
by in vivo or in vitro transcription of a cDNA clone,
originally produced from an alphavirus RNA and com-
prising an inserted exogenous DNA fragment encoding a
desired genetic trait.

Accordingly, the present invention is also related to
a DNA expression vector comprising a full-length or
partial cDNA complementary to alphavirus RNA or parts
thereof and located immediately downstream of the SP6

RNA polymerase promoter and having a 5'ATGG, a 5'GATGG or any other 5' terminus and a $TTTCCA_{69}ACTAGT$ or any other 3' terminus.

According to one aspect of the present invention portions of the viral cDNA are deleted, the deletions comprising the complete or part of the region(s) encoding the virus structural proteins, and the vector further comprises an integrated polylinker region, which may correspond to BamHI-SmaI-XmaI, inserted at a location which enables an exogenous DNA fragment encoding a foreign polypeptide or protein to be inserted into the vector cDNA for subsequent expression in an animal host cell.

According to another aspect of this invention, the vector is comprised of full-length cDNA wherein an exogenous DNA fragment encoding a foreign epitopic peptide sequence can be inserted into a region coding for the viral structural proteins.

It is appreciated that this cDNA clone with its exogenous DNA insert is very efficiently replicated after having been introduced into animal cells by transfection.

A very important aspect of the present invention is that it is applicable to a broad range of host cells of animal origin. These host cells can be selected from avian, mammalian, reptilian, amphibian, insect and fish cells. Illustrative of mammalian cells are human, monkey, hamster, mouse and porcine cells. Suitable avian cells are chicken cells, and as reptilian cells viper cells can be used. Cells from frogs and from mosquitoes and flies (Drosophila) are illustrative of amphibian and insecticidal cells, respectively. A very efficient virus vector/host cell system according to the invention is based on SFV/BHK cells, which will be discussed more in detail further below.

However, even though a very important advantage of the present DNA expression vector is that it is very

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efficient in a broad variety of animal cells it can
also be used in other eucaryotic cells and in pro-
caryotic cells.

The present invention is also related to a method to
produce transformed animal host cells comprising trans-
fection of the cells with the present RNA molecule or
with the present transcription vector comprised of cDNA
and carrying an exogenous DNA fragment. According to a
suitable embodiment of the invention, transfection is
produced by the above mentioned electroporation method,
a very high transfection rate being obtained.

A further suitable transformation process is based on
infection of the animal host cells with the above
mentioned infectious viral particles comprising the
present RNA molecule.

The transformed cells of the present invention can be
used for different purposes.

One important aspect of the invention is related to
use of the present transformed cells to produce a poly-
peptide or a protein by culturing the transformed cells
to express the exogenous RNA and subsequent isolation
and purification of the product formed by said exepres-
sion. The transformed cells can be produced by infec-
tion with the present viral particles comprising exo-
genous RNA encoding the polypeptide or protein as men-
tioned above, or by transfection with an RNA transcript
obtained by in vitro transcription of the present DNA
vector comprised of cDNA and carrying an exogenous DNA
fragment coding for the polypeptide or the protein.

Another important aspect of the invention is related
to use of the present transformed cells for the produc-
tion of antigens comprised of chimaeric virus particles
for use as immunizing component in vaccines or for
immunization purposes for in vivo production of
immunizing components for antisera production.

Accordingly, the present invention is also related to
an antigen consisting of a chimaeric alphavirus having

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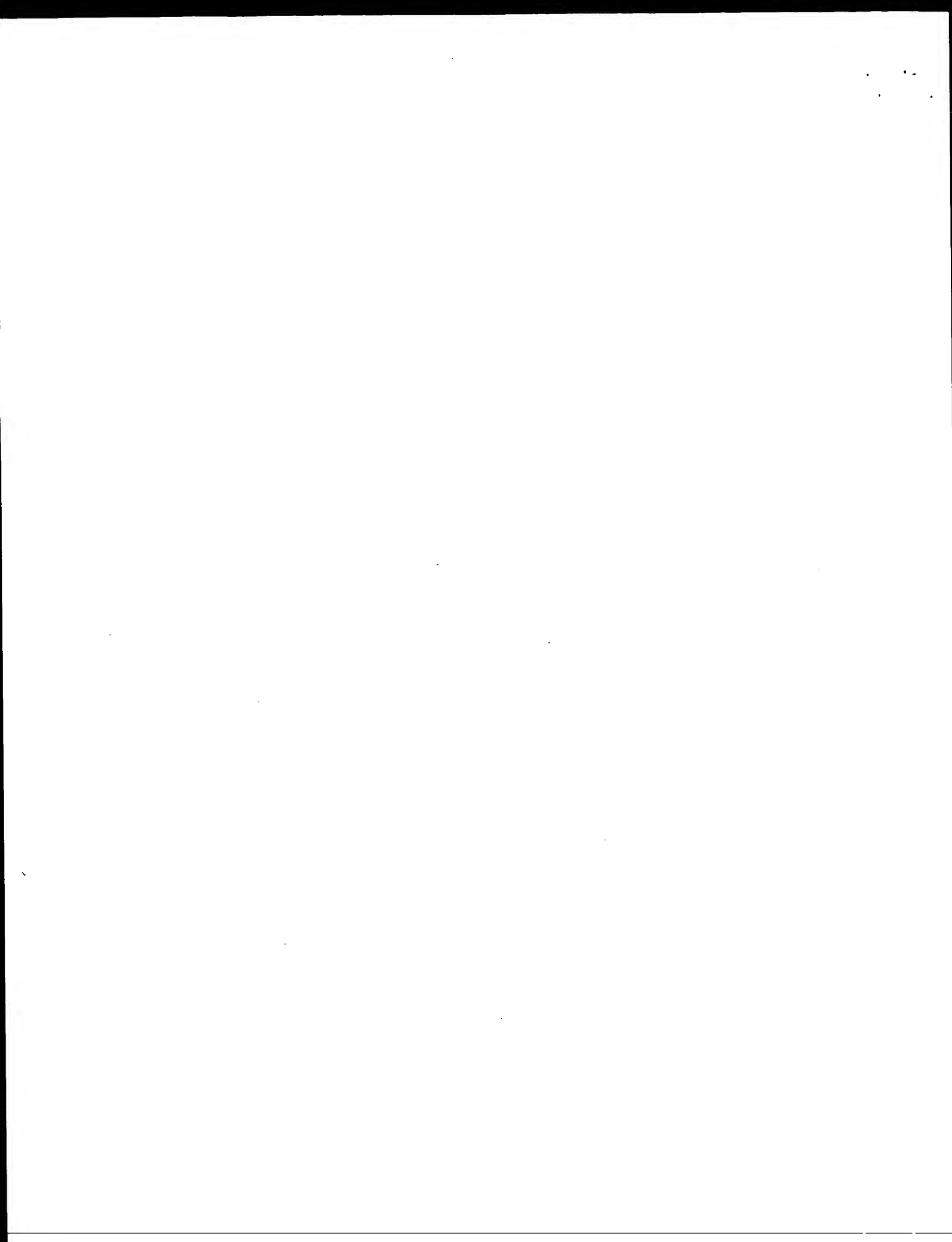
an exogenous epitopic peptide sequence inserted into its structural proteins.

Preferably, the chimaeric alphavirus is derived from SFV.

According to a suitable embodiment, the exogenous epitopic peptide sequence is comprised of an epitopic peptide sequence derived from a structural protein of a virus belonging to the immunodeficiency virus class inclusive of the human immunodeficiency virus types.

A further aspect of the invention is related to a vaccine preparation comprising the said antigen as immunizing component.

In said vaccine the chimaeric alphavirus is suitably attenuated by comprising mutations, such as the conditionally lethal SFV-mutation described before, amber (stop codon) or temperature sensitive mutations, in its genome.

For instance, if the chimaeric virus particles containing the afore mentioned conditional lethal mutation in its s tructural proteins (a defect to undergo a certain proteolytical cleavage in host cell during morphogenesis) is used as a vaccine then this is first activated by limited proteolytic treatment before given to the organism so that it can infect recipient cells. New chimaeric particles will be formed in cells infected with the activated virus but these will again be of the lethal phenotype and further spread of infection is not possible.

The invention is also concerned with a method for the production of the present antigen comprising

a) in vitro transcription of the cDNA of the present DNA vector carrying an exogenous DNA fragment encoding the foreign epitopic peptide sequence and transfection of animal host cells with the produced RNA transcript,

or

b) transfection of animal host cells with the said cDNA of the above step a),

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culturing the transfected cells and recovering the
chimaeric alphavirus antigen. Preferably, transfection
is produced by electroporation.

Still another aspect of the invention is to use a
recombinant virus containing exogenous RNA encoding a
polypeptide antigen for vaccination purpose or to pro-
duce antisera. In this case the recombinant virus or
the conditionally lethal variant of it is used to
infect cells in vivo and antigen production will take
place in the infectious cells and used for antigen
presentation to the immunological system.

According to another embodiment of the invention, the
present antigen is produced in an organism by using in
vivo infection with the present infectious particles
containing exogenous RNA encoding an exogenous epitopic
peptide sequence.

In the following, the present invention will be
illustrated more in detail with reference to the
Semliki Forest virus (SFV), which is representative for
the alphaviruses. This description can be more fully
understood in conjunction with the accompanying
drawings in which:

Fig. 1 is a schematic view over the main assembly and
disassembly events involved in the life cycle of the
Semliki Forest virus, and also shows regulation of the
activation of SFV entry functions by p62 cleavage and
pH;

Fig. 2 illustrates the use of translocation signals
during synthesis of the structural proteins of SFV;
top, the gene map of the 26S subgenomic RNA; middle,
the process·of membrane translocation of the p62, 6K
and E1 proteins; small arrows on the lumenal side
denote signal peptidase cleavages; at the bottom, the
characteristics of the three signal peptides are
listed;

Fig. 3 shows features that make SFV an excellent

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choice as an expression vector;

Fig. 4 A-C show the construction of full-length infectious clones of SFV; Fig. 4A shows a schematic restriction map of the SFV genome; primers used for initiating cDNA synthesis are indicated as arrows, and the cDNA inserts used to assemble the final clone are showed as bars; Fig. 4B shows plasmid pPLH211, i.e. the SP6 expression vector used as carrier for the full-length infectious clone of SFV, and the resulting plasmid pSP6-SFV4; Fig. 4C shows the structure of the SP6 promoter area of the SFV clone; the stippled bars indicate the SP6 promoter sequence, and the first necleotide to be transcribed is marked by an asterisk; underlined regions denote authentic SFV sequences;

Fig. 5 shows the complete nucleotide sequence of the pSP6-SFV4 RNA transcript as DNA (U = T) and underneath the DNA sequence, the amino acid sequence of the non-structural polyprotein and the structural polyprotein;

Fig. 6 shows an SFV cDNA expression system for the production of virus after transfection of in vitro made RNA into cells;

Fig. 7 shows the construction of the SFV expression vectors pSFV1-3 and of the Helper 1;

Fig. 8 shows the polylinker region of SFV vector plasmids pSFV1-3; the position of the promoter for the subgenomic 26S RNA is boxed, and the first nucleotide to be transcribed is indicated by an asterisk;

Fig. 9 is a schematic presentation of in vivo packaging of pSFV1-dhfr RNA into infectious particles using helper trans complementation; (dhfr means di-hydrofolate reductase)

Fig. 10 shows the use of trypsin to convert p62-containing noninfectious virus particles to infectious particles by cleavage of p62 to E2 and E3;

Fig. 11 shows the expression of heterologous proteins in BHK cells upon RNA transfection by electroporation; and

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Fig. 12 shows in its upper part sequences encompas-
sing the major antigenic site of SFV and the in vitro
made substitutions leading to a BamHI restriction endo-
nuclease site, sequences spanning the principal
neutralizing domain of the HIV gp120 protein, and the
HIV domain inserted into the SFV carrier protein E2 as
a BamHI oligonucleotide; and its lower part is a
schematic presentation of the SFV spike structure with
blow-ups of domain 246-251 in either wild type or
chimaeric form.

The alphavirus Semliki Forest virus (abbreviated SFV
in the following text) has for some 20 years been used
as model system in both virology and cell biology to
study membrane biosynthesis, membrane structure and
membrane function as well as protein-RNA interactions
(4, 5). The major reason for the use of SFV as such a
model is due to its simple structure and efficient
replication.

With reference to Fig. 1-3, in the following the SFV
and its replication are explained more in detail. In
essential parts, this disclosure is true also for the
other alphaviruses, such as the Sindbis virus, and many
of the references cited in this connection are indeed
directed to the Sindbis virus. SFV consists of an RNA-
containing nucleocapsid and a surrounding membrane
composed of a lipid bilayer and proteins, a regularly
arranged icosahedral shell of a protein called C pro-
tein forming the capsid inside which the genomic RNA is
packaged. The capsid is surrounded by the lipid bilayer
that contains three proteins called E1, E2, and E3.
These so-called envelope proteins are glycoproteins and
their glycosylated portions are on the outside of the
lipid bilayer, complexes of these proteins forming the
"spikes" that can be seen in electron micrographs to
project outward from the surface of the virus.

The SFV genome is a single-stranded 5'-capped and 3'-
polyadenylated RNA molecule of 11422 nucleotides (6,7).

It has positive polarity, i.e. it functions as an mRNA,
and naked RNA is able to start an infection when in-
troduced into the cytoplasm of a cell. Infection is
initiated when the virus binds to protein receptors on
5   the host cell plasma membrane, whereby the virions
become selectively incorporated into "coated pits" on
the surface of the plasma membrane, which invaginate to
form coated vesicles inside the cell, whereafter said
vesicles bearing endocytosed virions rapidly fuse with
10 organelles called endosomes. From the endosome, the
virus escapes into the cell cytosol as the bare nucleo-
capsid, the viral envelope remaining in the endosome.
Thereafter, the nucleocapsid is "uncoated" and, thus,
the genomic RNA is released. Referring now to Fig. 1,
15 infection then proceeds with the translation of the 5'
two-thirds of the genome into a polyprotein which by
self-cleavage is processed to the four nonstructural
proteins nsP1-4 (8). Protein nsP1 encodes a methyl
transferase which is responsible for virus-specific
20 capping activity as well as initiation of minus strand
synthesis (9, 10); nsP2 is the protease that cleaves
the polyprotein into its four subcomponents (11, 12);
nsP3 is a phosphoprotein (13, 14) of as yet unknown
function, and nsP4 contains the SFV RNA polymerase
25 activity (15, 16). Once the nsP proteins have been
synthesized they are responsible for the replication of
the plus strand (42S) genome into full-length minus
strands. These molecules then serve as templates for
the production of new 42S genomic RNAs. They also serve
30 as templates for the synthesis of subgenomic (26S) RNA.
This 4073 nucleotides long RNA is colinear with the
last one-third of the genome, and its synthesis is
internally initiated at the 26S promoter on the 42S
minus strands (17, 18).
35   The capsid and envelope proteins are synthesized in
different compartments, and they follow separate path-
ways through the cytoplasm, viz. the envelope proteins

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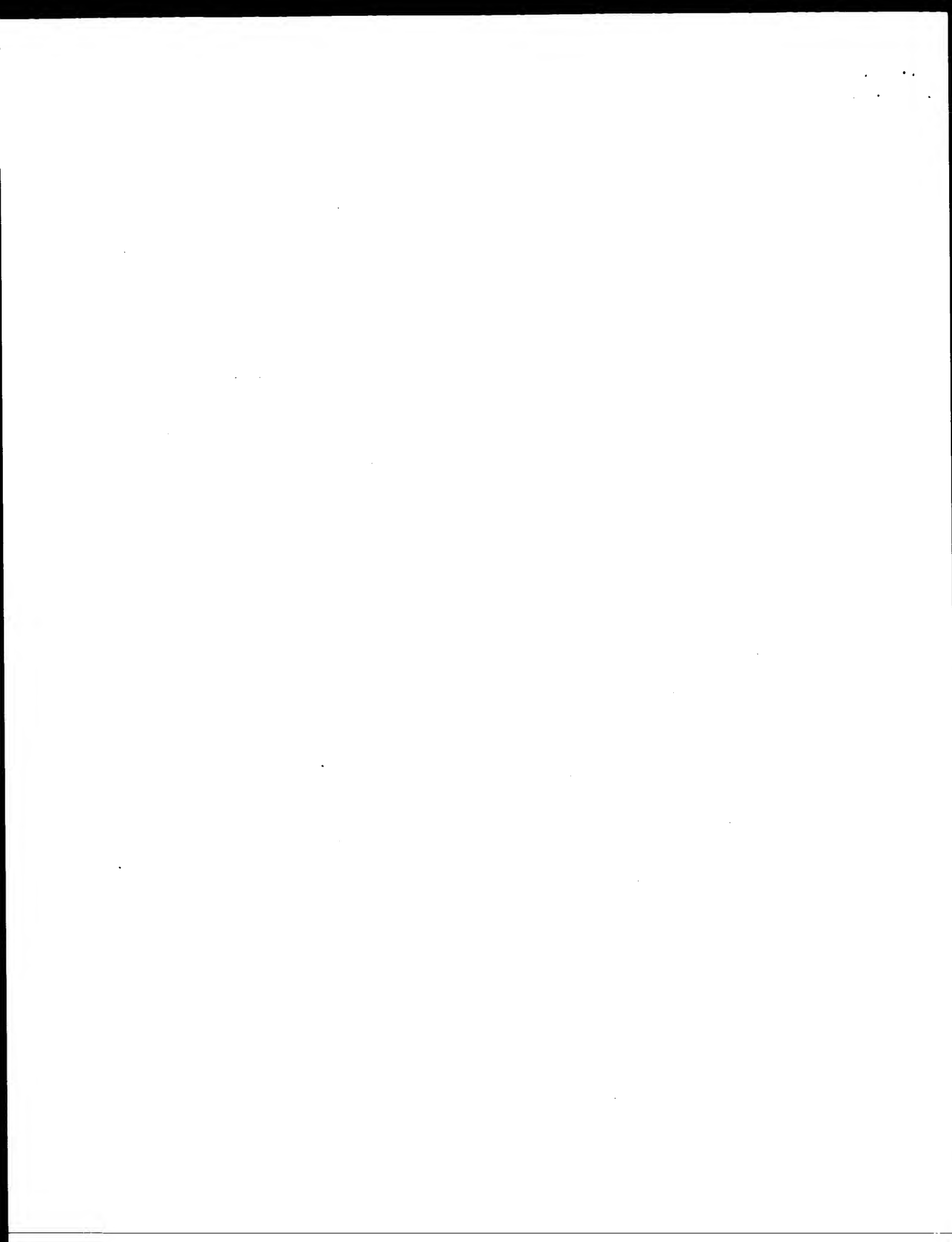
are synthesized by membrane-bound ribosomes attached to
the rough endoplasmic reticulum, and the capsid protein
is synthesized by free ribosomes in the cytosol. How-
ever, the 26S RNA codes for all the structural proteins
5   of the virus, and these are synthesized as a poly-
protein precursor in the order C-E3-E2-6K-E1 (19). Once
the capsid (C) protein has been synthesized it folds to
act as a protease cleaving itself off the nascent chain
(20, 21). The synthesized C proteins bind to the re-
10   cently replicated genomic RNA to form new nucleocapsid
structures in the cell cytoplasm.

The said cleavage reveals an N-terminal signal sequ-
ence in the nascent chain which is recognized by the
signal recognition particle targeting the nascent chain
15   - ribosome complex to the endoplasmic reticulum (ER)
membrane (22, 23), where it is cotranslationally
translocated and cleaved by signal peptidase to the
three structural membrane proteins p62 (precursor form
of E3/E2), 6K and E1 (24, 25). The translocational
20   signals used during the synthesis of the structural
proteins are illustrated in Fig. 2. The membrane pro-
teins undergo extensive posttranslational modifications
within the biosynthetic transport pathway of the cell.
The p62 protein forms a heterodimer with E1 via its E3
25   domain in the endoplasmic reticulum (26). This dimer is
transported out to the plasma membrane, where virus
budding occurs through spike nucleocapsid interactions.
At a very late (post-Golgi) stage of transport the p62
protein is cleaved to E3 and E2 (27), the forms that
30   are found in mature virions. This cleavage activates
the host cell binding function of the virion as well as
the membrane fusion potential of E1. The latter activi-
ty is expressed by a second, low-pH activation step
after the virus enters the endosomes of a new host cell
35   and is responsible for the release of the viral nucleo-
capsid into the cell cytoplasm (28-32). The mature
virus particles contain one single copy of the RNA

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genome encapsidated within 180 copies of the capsid
protein in a T=3 symmetry, and is surrounded by a lipid
bilayer carrying 240 copies of the spike trimer protein
consisting of E1+E2+E3 arranged in groups of three in a
T=4 symmetry (33).

The SFV entry functions are activated and regulated
by p62 cleavage and pH. More specifically, the p62-E1
heterodimers formed in the ER are acid resistant. When
these heterodimers are transported to the plasma mem-
brane via the Golgi complex the E1 fusogen cannot be
activated in spite of the mildly acidic environment,
since activation requires dissociation of the complex.
As is illustrated in Fig. 1, the released virus
particles contain E2E1 complexes. Since the association
between E2 and E1 is sensitive to acidic pH, during
entry of the virus into a host cell through endocytosis
the acidic milieu of the endosome triggers the dis-
sociation of the spike complex (E1 E2 E3) resulting in
free E1. The latter can be activated for the catalysis
of the fusion process between the viral and endosomal
membranes in the infection process as disclosed above.

As indicated in the preceding parts of the dis-
closure, the alphavirus system, and especially the SFV
system, has several unique features which are to
advantage in DNA expression systems. These are
summarized below with reference to Fig. 3.

1. Genome of positive polarity. The SFV RNA genome is
of positive polarity, i.e. it functions directly as
mRNA, and infectious RNA molecules can thus be obtained
by transcription from a full-length cDNA copy of the
genome.

2. Efficient replication. The infecting RNA molecule
codes for its own RNA replicase, which in turn drives
an efficient RNA replication. Indeed, SFV is one of the
most efficiently replicating viruses known. Within a
few hours up to 200.000 copies of the plus-RNAs are
made in a single cell. Because of the abundance of

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these molecules practically all ribosomes of the in-
fected cell will be enrolled in the synthesis of the
virus encoded proteins, thus overtaking host protein
synthesis (34), and pulse-labelling of infected cells
results in almost exclusive labelling of viral
proteins. During a normal infection 10^5 new virus
particles are produced from one single cell, which
calculates to at least 10^8 protein molecules encoded by
the viral genome (5).

3. Cytoplasmic replication. SFV replication occurs in
the cell cytoplasm, where the virus replicase trans-
cribes and caps the subgenomes for production of the
structural proteins (19). It would obviously be very
valuable to include this feature in a cDNA expression
system to eliminate the many problems that are encount-
ered in the conventional "nuclear" DNA expression
systems, such as mRNA splicing, limitations in trans-
cription factors, problems with capping efficiency and
mRNA transport.

4. Late onset of cytopathic effects. The cytopathic
effects in the infected cells appear rather late during
infection. Thus, there is an extensive time window from
about 4 hours after infection to up to 24 hours after
infection during which a very high expression level of
the structural proteins is combined with negligible
morphological change.

5. Broad host range. This phenomenon is probably a
consequence of the normal life cycle which includes
transmission through arthropod vectors to wild rodents
and birds in nature. Under laboratory conditions, SFV
infects cultured mammalian, avian, reptilian and insect
cells (35) (Xiong, et al, loc. cit.)

6. In nature SFV is of very low pathogenicity for
humans. In addition, the stock virus produced in tissue
culture cells is apparently apathogenic. By means of
specific mutations it is possible to create condi-
tionally lethal mutations of SFV, a feature that is of

great use to uphold safety when massproduction of virus stocks is necessary.

In the nucleotide and amino acid sequences the following abbreviations have been used in this specification:

Ala, alanine; lle, isoleucine; leu, leucine; Met, methionine; Phe, phenylalanine; Pro, proline; Trp, tryptophan; Val, valine; Asn, asparagine; Cys, cysteine; Gln, glutamine; Gly, glycine; Ser, serine; Thr, threonine; Tys, tyrosine; Arg, arginine; His, histidine; Lys, lysine; Asp, aspartic acid; Glu, glutamic acid; A, adenine; C, cytosine; G, guanine; T, thymine; U, uracil.

The materials and the general methodology used in the following examples are disclosed below.

1. Materials. Most restriction enzymes, DNA Polymerase I, Klenow fragment, calf intestinal phosphatase, T4 DNA ligase and T4 Polynucleotide kinase were from Boehringer (Mannheim, FRG). SphI, StuI and KpnI together with RNase inhibitor (RNasin) and SP6 Polymerase were from Promega Biotec (Madison, WI). Sequenase (Modified T7 polymerase) was from United States Biochemical (Cleveland, Ohio). Proteinase K was from Merck (Darmstadt, FRG). Ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides and the cap analogue $m^7G(5')ppp(5')G$ were from Pharmacia (Sweden). Oligonucleotides were produced using an Applied Biosystems synthesizer 380B followed by HPLC and NAP-5 (Pharmacia) purification. Spermidine, phenylmethylsulfonyl fluoride (PMSF), diethylpyrocarbonate (DEPC), bovine serum albumin (BSA), creatine phosphate and creatine phosphokinase were from Sigma (St. Louis, Mo). Pansorbin was from CalBiochem (La Jolla, CA). Agarose was purchased from FMC BioProducts (Rockland, Maine), and acrylamide from BioRad (Richmond, CA). L-[^{35}S]-methionine and α-[^{35}S]-dATP-α-S were from Amersham.

2. Virus growth and purification: BHK-21 cells were

grown in BHK medium (Gibco Life Technologies, Inc., New York) supplemented with 5 % fetal calf serum, 10 % tryptose phosphate broth, 10 mM HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) and 2 mM

5   glutamine. 90 % confluent monolayers were washed once with PBS and infected with SFV in MEM containing 0.2 % bovine serum albumin (BSA), 10 mM HEPES and 2 mM gluta-mine at a multiplicity of 0.1. Twenty-four hours post infection (p.i.) the medium was collected and cell

10 debris removed by centrifugation at 8,000 xg for 20 min at 4°C. The virus was pelleted from the medium by centrifugation at 26,000 rpm for 1.5 h in an SW28 rotor at 4°C. The virus was resuspended in TN containing 0.5 mM EDTA.

15   3. Metabolic labeling and immunoprecipitation. Con-fluent monolayers of BHK cells grown in MEM supplement-ed with 10 mM HEPES, 2 mM glutamine, 0.2 % BSA, 100 IU/mol of penicillin and 100 μg/ml streptomycin, were infected at a multiplicity of 50 at 37°C. After 1 h

20 p.i. the medium was replaced with fresh and growth continued for 3.5 h. The medium was removed and cells washed once with PBS and overlayed with methionine-free MEM containing 10 mM HEPES and 2 mM glutamine. After 30 min at 37°C the medium was replaced with the same con-

25 taining 100 μCi/ml of [^{35}S]methionine (Amersham) and the plates incubated for 10 min at 37°C. The cells were washed twice with labeling medium containing 10X excess methionine and then incubated in same medium for various times. The plates were put on ice, cells washed

30 once with ice-cold PBS and finally lysis buffer (1 % NP-40 - 50 mM Tris-HCl, pH 7.6 - 150 mM NaCl - 2 mM EDTA) containing 10 μg/ml PMSF (phenylmethylsulfonyl fluoride) was added. Cells were scraped off the plates, and nuclei removed by centrifugation at 6,000 rpm for 5

35 min at 4°C in an Eppendorf centrifuge. Immunoprecipita-tions of proteins was performed as described (31). Briefly, antibody was added to lysate and the mixture

kept on ice for 30 min. Complexes were recovered by
binding to Pansorbin for 30 min on ice. Complexes were
washed once with low salt buffer, once with high salt
buffer, and once with 10 mM Tris-HCl, pH 7.5, before
heating with gel loading buffer. To precipitate dhfr,
SDS was added to 0.1 % and the mixture heated to 95°C
for 2 min followed by addition of 10 volumes of lysis
buffer. Anti-E1 [8.139], anti-E2 [5.1] (36), and anti-C
[12/2] (37) monoclonals have been described. The human
transferrin receptor was precipitated with the mono-
clonal antibody OKT-9 in ascites fluid. This prepara-
tion was provided by Thomas Ebel at our laboratory
using a corresponding hybridoma cell line obtained from
ATCC (American Typ Culture Collection) No CRL 8021.
Polyclonal rabbit anti-mouse dhfr was a kind gift from
E. Hurt (European Molecular Biology Laboratory, Heidel-
berg, FRG) and rabbit anti-lysozyme has been described
(38).

4. Immunofluorescence. To perform indirect immuno-
fluorescence, infected cell monolayers on glass cover-
slips were rinsed twice with phosphate-buffered saline
(PBS) and fixed in -20°C methanol for 6 min. After
fixation, the methanol was removed and the coverslip
washed 3 times with PBS. Unspecific antibody binding
was blocked by incubation at room temperature with PBS
containing 0.5 % gelatin and 0.25 % BSA. The blocking
buffer was removed and replaced with same buffer con-
taining primary antibody. After 30 min at room tempera-
ture the reaction was stopped by washing 3 times with
PBS. Binding of secondary antibody (FITC-conjugated
sheep anti-mouse [BioSys, Compiégne, France]) was done
as for the primary antibody. After 3 washes with PBS
and one rinse with water the coverslip was allowed to
dry before mounting in Moviol 4-88 (Hoechst, Frankfurt
am Main, FRG) containing 2.5 % DABCO (1,4-diazobicyclo-
[2.2.2]-octane).

5. DNA procedures. Plasmids were grown in Escherichia

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coli DH5α (Bethesda Research Laboratories) [recA endA1
gyrA96 thi1 hsdR17 supE44 relA1 Δ(lacZYA-argF)U169
φ80dlacZΔ(M15)]. All basic DNA procedures were done
essentially as described (39). DNA fragments were iso-
5   lated from agarose gels by the freeze-thaw method (40)
including 3 volumes of phenol during the freezing step
to increase yield and purity. Fragments were purified
by benzoyl-naphthoyl-DEAE (BND) cellulose (Serva Fein-
biochemica, Heidelberg, FRG) chromatography (41).
10   Plasmids used for production of infectious RNA were
purified by sedimentation through 1 M NaCl followed by
banding in CsCl (39). In some cases plasmids were puri-
fied by Qiagen chromatography (Diagen Gmbh, Düsseldorf,
FRG).
15   6. Site-directed oligonucleotide mutagenesis. For
oligonucleotide mutagenesis, relevant fragments of the
SFV cDNA clone were subcloned into M13mp18 or mp 19
(42) and transformed (43) into DH5αFIQ [endA1 hsdR1
supE44 thi1 recA1 gyrA96 relA1 φ80dlacΔ(M15) Δ(lacZYA-
20   argF)U169/F'proAB lacl^q lacZΔ(M15) Tn 5] (Bethesda
Research Laboratories). RF DNA from these constructs
was transformed into RZ1032 (44) [Hfr KL16 dut1 ung1
thi1 relA1 supE44 zbd279:Tn10.], and virus grown in the
presence of uridine to incorporate uracil residues into
25   the viral genome. Single stranded DNA was isolated by
phenol extraction from PEG precipitated phage. Oligo-
nucleotides were synthesized on an Applied Biosystems
380B synthesizer and purified by gel filtration over
NAP-5 columns (Pharmacia). The oligonucleotides
30   5'-CGGCCAGTGAATTCTGATTGGATCCCGGGTAATTAATTGAATTACATCCC-
TACGCAAACG, 5'-GCGCACTATTATAGCACCGGCTCCCGGGTAATTAATT-
GACGCAAACGTTTTACGGCCGCCGG and 5'-GCGCACTATTATAGCACCATG-
GATCCGGGTAATTAATTGACGTTTTACGGCCGCCGGTGGCG were used to
insert the new linker sites [BamHI-SmaI-XmaI] into the
35   SFV cDNA clone. The oligonucleotides 5'-CGGCGGTCCTA-
GATTGGTGCG and 5'-CGCGGGCGCCACCGGCGGCCG were used as
sequencing primers (SP1 and SP2) up- and downstream of

the polylinker site. Phosphorylated oligonucleotides were used in mutagenesis with Sequenase (Unites States Biochemicals, Cleveland, Ohio) as described earlier (44, 45). In vitro made RF forms were transformed into DH5αF'IQ and the resulting phage isolates analyzed for the presence of correct mutations by dideoxy sequencing according to the USB protocol for using Sequenase. Finally, mutant fragments were reinserted into the full-length SFV cDNA clone. Again, the presence of the appropriate mutations was verified by sequencing from the plasmid DNA. Deletion of the 6K region has been described elsewhere.

7. In vitro transcription. SpeI linearized plasmid DNA was used as template for in vitro transcription. RNA was synthesized at 37°C for 1 h in 10-50 μl reactions containing 40 mM Tris-HCl (pH 7.6), 6 mM spermidine-HCl, 5 mM dithiothreitol (DTT), 100 μg/ml of nuclease free BSA, 1 mM each of ATP, CTP and UTP, 500 μM of GTP, 1 unit/μl of RNasin and 100-500 units/ml of SP6 RNA polymerase. For production of capped transcripts (46), the analogs $m^7G(5')ppp(5')G$ or $m^7G(5')ppp(5')A$ were included in the reaction at 1 mM. For quantitation of RNA production, trace amounts of $[\alpha-^{32}P]$-UTP (Amersham) was included in the reactions and incorporation measured from trichloroacetic acid precipitates. When required, DNA or RNA was digested at 37°C for 10 min by adding DNase 1 or RNase A at 10 units/μg template or 20 μg/ml respectively.

8. RNA transfection. Transfection of BHK monolayer cells by the DEAE-Dextran method was done as described previously (47). For transfection by electroporation, RNA was added either directly from the in vitro transcription reaction or diluted with transcription buffer containing 5 mM DTT and 1 unit/μl of RNasin. Cells were trypsinized, washed once with complete BHK-cell medium and once with ice-cold PBS (without $MgCl_2$ and $CaCl_2$) and finally resuspended in PBS to give 10^7 cells/ml. Cells

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were either used directly or stored (in BHK medium) on
ice over night. For electroporation, 0.5 ml of cells
were transferred to a 0.2 cm cuvette (BioRad), 10-50 μl
of RNA solution added and the solution mixed by invert-
ing the cuvette. Electroporation was performed at room
temperature by two consecutive pulses at 1.5 kV/25 μF
using a BioRad Gene Pulser apparatus with its pulse
controller unit set at maximum resistance. After in-
cubation for 10 min, the cells were diluted 1:20 in
complete BHK-cell medium and transferred onto tissue
culture plates. For plaque assays, the electroporated
cells were plated together with about $3x10^5$ fresh cells
per ml and incubated at 37°C for 2 h, then overlayed
with 1.8 % low melting point agarose in complete BHK-
cell medium. After incubation at 37°C for 48 h, plaques
were visualized by staining with neutral red.

9. Gel electrophoresis. Samples for sodium dodecyl
sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)
were prepared and run on 12 % separating gels with a 5
% stacking gel as previously described (48). For re-
solving the 6K peptide, a 10 % - 20 % linear acrylamide
gradient gel was used. Gels were fixed in 10 % acetic
acid - 30 % methanol for 30 min before exposing to
Kodak XAR-5 film. When a gel was prepared for fluoro-
graphy (49), it was washed after fixation for 30 min in
30 % methanol and then soaked in 1M sodium salicylate -
30 % methanol for 30 min before drying. Nucleic acids
were run on agarose gels using 50 mM Tris-borate - 2.5
mM Na_2EDTA as buffer. For staining 0.2 μg/ml of ethidium
bromide was included in the buffer and gel during the
run.

Example 1

In this example a full-length SFV cDNA clone is
prepared and placed in a plasmid containing the SP6 RNA
polymerase promoter to allow in vitro trancription of
full-length and infectious transcripts. This plasmid
which is designated pSP6-SFV4 has been deposited on 28

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NOV 1991 at PHLS Centre for Applied Microbiology &
Research

European Collection of Animal Cell Cultures, Porton
Down, Salisbury, Wiltshire, U.K:, and given the pro-
visional accession number 91112826.

As illustrated in Fig. 4A-C the strategy for con-
struction the SFV clone was to prime cDNA synthesis on
several positions along the template RNA downstream of
suitable restriction endonuclease sites defined by the
known nucleotide sequence of the SFV RNA molecule.
Virus RNA was isolated by phenol-chloroform extraction
from purified virus (obtainable among others from the
Arbovirus collection in Yale University, New Haven,
USA) and used as template for cDNA synthesis as
previously described (50). First strand synthesis was
primed at three positions, using 5'-TTTCTCGTAGTTCTCCTC-
GTC as primer-1 (SFV coordinate 2042-2062) and 5'-GTTA-
TCCCAGTGGTTGTTCTCGTAATA as primer-2 (SFV coordinate
3323-3349) and an oligo-dT$_{12-18}$ as primer -3 (3' end of
SFV) Fig. 4A).

Second strand synthesis was preceded by hybridization
of the oligonucleotide 5'-ATGGCGGATGTGTGACATACACGACGCC
(identical to the 28 first bases of the genome sequence
of SFV) to the first strand cDNA. After completion of
second strand synthesis cDNA was trimmed and in all
cases except in the case of the primer-1 reaction, the
double-stranded adaptor 5'-AATTCAAGCTTGCGGCCGCACTAGT /
GTTCGAACGCCGGCGTGATCA-3' (5'-sticky-EcoRI-HindIII-NotI-
XmaIII-SpeI-blunt-3') was added and the cDNa cloned
into EcoRl cleaved pTZ18R (Pharmacia, Sweden) as
described (51). The cloning of the 5' end region was
done in a different way. Since SFV contains a HindIII
site at position 1947, cDNA primed with primer-1 should
contain this area and therefore HindIII could be used
to define the 3' end of that cDNA. To obtain a restric-
tion site at the very 5' end of the SFV, cDNA was
cloned into SmaI-HindIII cut pGEM1 (Promega Biotec.,

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Madison, WI). Since the SFV genome starts with the
sequence 5'-ATGG, ligation of this onto the blunt CCC-
3' end of the SmaI site created an NcoI site C'CATGG.
Although the SFV sequence contains 3 NcoI sites, none
5   of these are within the region preceding the HindIII
site, and thus these 5' end clones could be further
subcloned as NcoI-HindIII fragments into a vector
especially designed for this purpose (see below). The
original cDNA clones in pGEM1 were screened by restric-
10   tion analysis and all containing inserts bigger than
1500 bp were selected for further characterization by
sequencing directly from the plasmid into both ends of
the insert, using SP6 or T7 sequencing primers. The SFV
5'-end clones in pTZ18R were sequenced using lac
15   sequencing primers. To drive in vitro synthesis of SFV
RNA the SP6 promoter was used. Cloning of the SFV 5'
end in front of this promoter without adding too many
foreign nucleotides required that a derivative of pGEM1
had to be constructed. Hence, pGEM1 was opened at EcoR1
20   and Bal31 deletions were created, the DNA blunted with
T4 DNA polymerase and an NcoI oligonucleotide (5'-
GCCATGGC) added. The clones obtained were screened by
colony hybridization (39) with the oligonucleotide 5'-
GGTGACACTATAGCCATGGC designed to pick up (at suitable
25   stringency) the variants that had the NcoI sequence
immediately at the transcription initiation site of the
SP6 promoter (G underlined). Since the Bal31 deletion
had removed all restriction sites of the multicloning
site of the original plasmid, these were restored by
30   cloning a PvuI-NcoI fragment from the new variant into
another variant of pGEM1 (pDH101) that had an NcoI site
inserted at its HindIII position in the polylinker.
This created the plasmid pDH201. Finally, the adaptor
used for cloning the SFV cDNA was inserted into pDH201
35   between the EcoRI and PvuII sites to create plasmid
pPLH211 (Fig. 4B). This plasmid was then used as
recipient for SFV cDNA fragments in the assembly of the

29.

full-length clone by combining independent overlapping
subclones using these sites. The fragments and the
relevant restriction sites used to assemble the full-
length clone, pSP6-SFV4, are depicted in (Fig. 4A). For
the 5'-end, the selected fragment contained the proper
SFV sequence 5'-ATGG, with one additional G-residue in
front. When this G-residue was removed it reduced
transcription efficiency from SP6 but did not affect
infectivity of the in vitro made RNA. Thus, the clone
used for all subsequent work contains the G-residue at
the 5' end. For the 3'-end of the clone, a cDNA
fragment containing 69 A-residues was selected. By
inclusion of the unique SpeI site at the 3'-end of the
cDNa, the plasmid can be linearized to allow for runoff
transcription in vitro giving RNA-carrying 70 A-
residues. Fig. 4C shows the 5' and 3' border sequences
of the SFV cDNA clone. The general outline how to
obtain and demonstrate infectivity of the full-length
SFV RNA is depicted in Fig. 6. The complete nucleotide
sequence of the pSP6-SFV4 SP6 transcript together with
the amino acid sequences of the nonstructural and the
structural polyproteins is shown in Fig. 5.

Typically, about 5 μg of RNA per 100 ng of template
was obtained using 10 units of polymerase, but the
yield could be increased considerably by the use of
more enzyme. The conditions slightly differ from those
reported earlier for the production of infectious tran-
scripts of alphaviruses (52) (47). A maximum production
of RNA was obtained with rNTP concentrations at 1 mM.
However, since infectivity also is dependent on the
presence of a 5'cap structure optimal infectivity was
obtained when the GTP concentration in the transcrip-
tion reaction was halved. This drop had only a marginal
effect on the amounts of RNA produced but raised the
specific infectivity by a factor of 3 (data not shown).

The cDNA sequence shown in Fig. 5 has been used in
the following examples. However, sequences having one

30

or a few nucleotides, which differ from those shown in
Fig. 5, could also be useful as vectors, even if these
might be less efficient as illustrated above with the
SFV cDNA sequence lacking the first 5'-G nucleotide in
5   Fig. 5.

Example 2.

In this example the construction of SFV DNA expres-
sion vectors is disclosed.

The cDNA clone coding for the complete genome of SFV
10   obtained in Example 1 was used to construct a SFV DNA
expression vector by deletion of the coding region of
the 26S structural genes to make way for heterologous
inserts. However, the nonstructural coding region,
which is required for the production of the nsP1-4
15   replicase complex is preserved. RNA replication is
dependent on short 5' (nt 1-247) (53, 54, 55) and 3'
(nt 11423-11441) sequence elements (56, 57), and there-
fore, also these had to be included in the vector con-
struct, as had the 26S promoter just upstream of the C
20   gene (17, 18).

As is shown in Fig. 7, first, the XbaI (6640)-NsiI
(8927) fragment from the SFV cDNA clone pSP6-SFV4 from
Example 1 was cloned into pGEM7Zf(+) (Promega Corp., WI,
USA) (Step A). From the resulting plasmid, pGEM7Zf(+)-
25   SFV, the EcoRI fragment (SFV coordinates 7391 and
88746) was cloned into M13mp19 to insert a BamHI - XmaI
- SmaI polylinker sequence immediately downstream from
the 26S promoter site using site-directed mutagenesis
(step B). Once the correct mutants had been verfied by
30   sequencing from M13 ssDNA (single stranded), the EcoRI
fragments were reinserted into pGEM7Zf(+)-SFV (step C)
and then cloned back as XbaI-Nsλ fragments into pSP6-
SFV4 (step D). To delete the major part of the cDNA
region coding for the structural proteins of SFV, these
35   plasmids were then cut with AsuII (7783) and NdeI
(11033), blunted using Klenow fragment in the presence
of all four nucleotides, and religated to create the

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final vectors designated pSFV1, pSFV2 and pSFV3,
respectively (step E). The vectors retain the promoter
region of the 26S subgenomic RNA and the last 49 amino
acids of the E1 protein as well as the complete non-
coding 3' end of the SFV genome.

In the vectors the subgenomic (26S) protein coding
portion has been replaced with a polylinker sequence
allowing the insertional cloning of foreign cDNA
sequences under the 26S promoter. As is shown in Fig. 8
these three vectors have the same basic cassette in-
serted downstream from the 26S promoter, i.e. a poly-
linker (BamHI-SmaI-XmaI) followed by a translational
stop-codons in all three reading frames. The vectors
differ as to the position where the polylinker cassette
has been inserted. In pSFV1 the cassette is situated 31
bases downstream of the 26S transcription initiation
site. The initiation motive of the capsid gene transla-
tion is identical to the consensus sequence (58).
Therefore, this motive has been provided for in pSFV2,
where it is placed immediately after the motive of the
capsid gene. Finally, pSFV3 has the cassette placed
immediately after the initiation codon (AUG) of the
capsid gene. Sequencing primers (SP) needed for
checking both ends of an insert have been designed to
hybridize either to the 26S promoter region
(SP1), or to the region following the stop codon
cassette (SP2).

Note that the 26S promoter overlaps with the 3'-end
of the nsP4 coding region. For pSFV2, the cloning site
is positioned immediately after the translation initia-
tion site of the SFV capsid gene. For pSFV3, the
cloning site is positioned three nucleotides further
downstream, i.e. immediately following to the initial
AUG codon of the SFV capsid gene. The three translation
stop codons following the polylinker are boxed. The
downstream sequencing primer (SP1) overlaps with the
26S promoter, and the upstream sequencing primer (Sp2)

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overlaps the XmaIII site.

Example 3

In this example an in vivo packaging system encompassing helper virus vector constructs is prepared. .

The system allows SFV variants defective in structural protein functions, or recombinant RNAs derived from the expression vector construct obtained in Example 2, to be packaged into infectious virus particles. Thus, this system allows recombinant RNAs to be introduced into cells by normal infection. The helper vector, called pSFV-Helper1, is constructed by deleting the region between the restriction endonuclease sites AccI (308) and AccI (6399) of pSP6-SFV4 obtained in Example 1 by cutting and religation as shown in Fig. 7, step F. The vector retains the 5' and 3' signals needed for RNA replication. Since almost the complete nsP region of the Helper vector is deleted, RNA produced from this construct will not replicate in the cell due to the lack of a functional replicase complex. As is shown in Fig. 9, after transcription in vitro of pSFV1-recombinant and helper cDNAs, helper RNA is cotransfected with the pSFV1 - recombinant derivative, the helper construct providing the structural proteins needed to assemble new virus particles, and the recombinant providing the nonstructural proteins needed for RNA replication, SFV particles comprising recombinant genomes being produced. The cotransfection is preferably produced by electroporation as is disclosed in Example 6 and preferably BHK cells are used as host cells.

To package the RNA a region at the end of nsP1 is required, an area which has been shown to bind capsid protein (57, 59). Since the Helper lacks this region, RNA derived from this vector will not be packaged and hence, transfections with recombinant and Helper produces only virus particles that carry recombinant-derived RNA. It follows that these viruses cannot be

passaged further and thus provide a one-step virus stock. The advantage is that infections with these particles will not produce any viral proteins.

Example 4

This example illustrates the construction of variants of the full-length SFV cDNA clone from Example 1 that allow insertion of foreign DNA sequences encoding foreign epitopes, and the production of recombinant (chimaeric) virus carrying said foreign epitopes as integral parts of the p62, E2 or E1 spike proteins.

To this end, a thorough knowledge of the function, topology and antigenic structure of the E2 and E1 envelope proteins has been of the essence. Earlier studies on the pathogenicity of alphaviruses have shown that antibodies against E2 are type-specific and have good neutralizing activity while those against E1 generally are group-specific and are nonneutralizing (5). However, not until recently have antigenic sites of the closely related alphaviruses SFV, Sindbis, and Ross River been mapped and correlated to the level of amino acid sequence (60, 61, 62, 63). These studies have shown that the most dominant sites in question are at amino acid positions 216, 234 and 246-251 of the SFV E2 spike protein. Interestingly, these three sites are exactly the same as the ones predicted by computer analysis. In the present example domain 246-251 was used, since this area has a highly conserved structure and hydropathy profile within the group of alpha-viruses. Insertion of a gene encoding a foreign epitope into the 246-251 region of the pSP6-SFV4 p62 protein yields particles with one new epitope on each hetero-dimer, i.e. 240 copies.

To create a unique restriction endonuclease site that would allow specific insertion of foreign epitopes into the E2 portion of the SFV genome, a BamHI site was inserted by site directed mutagenesis using the oligo-nucleotide 5'-GATCGGCCTAGGAGCCGAGAGCCC.

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Example 5

In this example a conditionally lethal variant of SFV
is constructed from the SFV cDNA obtained in Example 1,
which variant carries a mutation in the p62 protein
resulting in a noncleavable from of said protein, with
the result that this variant as such cannot infect new
host cells, unless first cleaved with exogenously added
protease.

As illustrated in Fig. 10, this construct can be
advantageously used as a vaccine carrier for foreign
epitopes, since this form of the virus cannot enter new
host cells although assembled with wild type efficiency
in transfected cells. The block can be overcome by
trypsin treatment of inactive virus particles. This
converts the particle into a fully entry-competent form
which can be used for amplification of this virus
variant stock.

Once activated the SFV variant will enter cells
normally through the endocytic pathway and start infec-
tion. Viral proteins will be made and budding takes
place at the plasma membrane. However, all virus
particles produced will be of inactive form and the
infection will thus cease after one round of replica-
tion. The reason for the block in infection proficiency
is a mutation which has been introduced by site
directed mutagenesis into the cleavage site of p62.
This arginine to leucine substitution (at amino acid
postion 66 of the E3 portion of the p62 protein)
changes the consensus features of the cleavage site so
that it will not be recognized by the host cell pro-
teinase that normally cleaves the p62 protein to the E2
and E3 polypeptides during transport to the cell
surface. Instead, only exogenously added trypsin will
be able to perform this cleavage, which in this case
occurs at the arginine residue 65 immediately preceding
the original cleavage site. As this cleavage regulates
the activation of the entry function potential of the

virus by controlling the binding of the entry spike
subunit, the virus particle carrying only uncleaved p62
will be completely unable to enter new host cells.

The creation of the cleavage deficient mutation E2
has been described earlier (29). An AsuII - Nsλ
fragment spanning this region was then isolated and
cloned into the full-length cDNA clonepSP6-SFV4.

Example 6

In this example transfection of BHK cells with SFV
RNA molecules transcribed in vitro from full-length
cDNA from Example 1 or variants thereof or the SFV
vectors from Example 2, which comprise exogenous DNA,
is disclosed. The transfection is carried out by
electroporation which is shown to be very efficient at
optimized conditions.

BHK cells were transfected with the above SFV RNA
molecules by electroporation and optimal conditions
were determined by varying parameters like temperature,
voltage, capacitance, and number of pulses. Optimal
transfection was obtained by 2 consecutive pulses of
1.5 kV at 25 μF, under which negligible amounts of
cells were killed. It was found that it was better to
keep the cells at room tempeature than at 0°C during
the whole procedure. Transfection by electroporation
was also measured as a function of input RNA. As
expected, an increase in transfection frequency was not
linearly dependent on RNA concentration, and about 2 μg
of cRNA were needed to obtain 100 % transfection.

On comparison with conventional transfection, this is
a great improvement. For example, with DEAE-Dextran
transfection optimally, only 0.2 % of the cells were
transfected.

Example 7

This example illustrates heterologous gene expression
driven by the SFV vector, pSFV1 from Example 2, for
genes encoding the 21 kD cytoplasmic mouse dihydro-
folate reductase (dhfr), the 90 kD membrane protein

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human transferrin receptor (TR), and finally the 14 kD
secretory protein chicken lysozyme. The dhfr gene was
isolated from pGEM2-dhfr (64) as a BamHI-HindIII
fragment blunted with Klenow fragment and inserted into

5   SmaI-cut pSFV1. The transferrin receptor gene was first
cloned from pGEM1-TR (64, 65) as an XbaI-EcoRI fragment
into pGEM7ZF(+) and subsequently from there as a BamHI
fragment into pSFV1. Finally, a BamHI fragment from
pGEM2 carrying the lysozyme gene (21) was cloned into

10   pSFV1.

To study the expression of the heterologous proteins,
in vitro-made RNA of the dhfr and TR constructs was
electroporated into BHK cells. RNA of wild type SFV was
used as control. At different time points post electro-

15   poration (p.e.) cells were pulse-labeled for 10 min
followed by a 10 min chase, whereafter the lysates were
analyzed by gel electrophoresis and autoradiography.
The results are shown in Figure 11. More specifically,
BHK cells were transfected with RNAs of wild type SFV,

20   pSFV1-dhfr, and pSFV1-TR, pulse-labeled at 3, 6, 9, 12,
15 and 24 h p.e. Equal amounts of lysate were run on a
12 % gel. The 9 h sample was also used in immunopreci-
pitation (IP) of the SFV, the dhfr and the transferrin
receptor proteins. Cells transfected with pSFV1-

25   lysozyme were pulse-labeled at 9 h p.e. and then chased
for the times (hours) indicated. An equal portion of
lysate or medium was loaded on the 13,5 % gel. IP
represents immunoprecipitation from the 1 h chase
lysate sample. The U-lane is lysate of labeled but

30   untransfected cells. At 3 h p.e.hardly any exogenous
proteins were made, since the incoming RNA starts with
minus strand synthesis which does not peak until about
4-5 h p.e. (5). At this time point, almost all labeled
proteins were of hos origin. In contrast, at 6 h p.e.

35   the exogenous proteins were synthesized with great
efficiency, and severe inhibition of host protein syn-
thesis was evident. This was even more striking at 9 h

p.e., when maximum shut down had been reached.
Efficient production of the heterologous proteins con-
tinued up to 24 h p.e., after which production slowed
down (data not shown), indicating that the cells had
entered a stationary phase.

.Since chicken lysozyme is a secretory protein, its
expression was analyzed both from cell lysates and from
the growth medium. Cells were pulse-labeled at 9 h p.e.
and then chased up to 8 h. The results are shown in
Fig. 11. Although lysozyme was slowly secreted, almost
all labeled material was secreted to the medium during
the chase.

Example 8

This example illustrates the present in vivo
packaging system.

In vitro-made RNA of pSFV1-TR was mixed with Helper
RNA at different ratios and these mixtures were co-
transfected into BHK cells. Cells were grown for 24 h
after which the culture medium was collected and the
virus particles pelleted by ultracentrifugation. The
number of infectious units (i.u.) was determined by
immunofluorescence. It was found that a 1:1 ratio of
Helper and recombinant most efficiently produced in-
fectious particles, and on the average 5 x 10^6 cells
yielded 2.5 x 10^9 i.u. The infectivity of the virus
stock was tested by infecting BHK cells at different
multiplicities of infection (m.o.i.). In Fig. 11 the
results for expression of human transferrin receptor in
BHK cells after infection by such in vivo packaged
particles carrying pSFV1-TR recombinant RNA is shown to
the lower right. 200 μl of virus diluted in MEM (in-
cluding 0,5'% BAS and 2 mM glutamine) was overlaid on
cells to give m.o.i. values ranging from 5 to 0.005.
After 1 h at 37°C, complete BHK medium was added and
growth continued for 9 h, at which time a 10 min pulse
(100 μCi ^{35}S-methionine/ml) and 10 min chase was
performed, and the cells dissolved in lysis buffer. 10

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μl out of the 300 μl lysate (corresponding to 30,000
cells) was run on the 10 % gel, and the dried gel was
exposed for 2 h at -70°C. Due to the high expression
level, only 3,000 cells are needed to obtain a distinct
band on the autoradiograph with an over night exposure.

Thus, it was found that efficient protein production
and concomitant hos protein shut-off occurred at about
1 i.u. per cell. Since one SFV infected cell produces
on the average 10^8 capsid protein molecules, it follows
that a virus stock produced from a single electropora-
tion can be used to produce 10^{17} protein molecules
equaling about 50 mg of protein.

From the foregoing experimental results it is obvious
that the present invention is related to very useful
and efficient expression system which lacks several of
the disadvantages of the hitherto existing expression
system. The major advantages of the present system are
shortly summarized as follows:

(1)   High titre recombinant virus stocks can be produced
        in one day by one transfection experiment. There is
        no need for selection/screening, plaque purifica-
        tion and amplification steps. This is appreciated
        since an easy production of recombinant virus is
        especially important in experiments where the
        phenotypes of large series of mutants have to be
        characterized.

(2)   The recombinant virus stock is free from helper
        virus since only the recombinant genome but not the
        helper genome contains a packaging signal.

(3)   The recombinant virus can be used to infect the
        recombinant genome in a "natural" and nonleakey way
        into a large variety of cells including insect and
        most higher euoaryotic cell types. Such a wide host
        range is very useful for an expressions system

especially when cell-type-specific posttranslatio-
nal modification reactions are required for the
activity of the expressed protein.

(4)   The level of protein expression obtained is
extremely high, the level corresponding to those of
the viral proteins during infection. There is also
a host cell protein shut-off which makes it
possible to follow the foreign proteins clearly in
cell lysates without the need for antibody mediated
antigen concentration. This will facilitate DNA
expression experiments in cell biology considerab-
ly. Furthermore, problems of interference by the
endogenous counter part to an expressed protein
(i.e. homo-oligomerization reactions) can be
avoided.

Example 9

This example illustrates epitope carriers.

A very important example where vaccine development is
of the utmost importance concerns the acquired immuno-
deficiency syndrome (AIDS) caused by the human immuno-
deficiency virus HIV-1 (66, 67). Sofar, all attempts to
produce an efficient vaccine against HIV-1 have failed,
although there was a very recent report that vaccina-
tion with disrupted SIV-1 (Simian immunodeficiency
virus) to a certain extent may give protection against
infections of that virus (68). However, development of
safe and effective vaccine against HIV-1 will be very
difficult due to the biological properties of the
virus. In the present exampel one epitope of HIV-1 was
inserted into an antigenic domain of the E2 protein of
SFV. The epitope used is located in glycoprotein gp120
of HIV-1, spanning amino acids 309-325. This forms the
variable loop of HIV-1 and is situated immediately
after an N-glycosylated site.

A chimaera was constructed where the 309-325 epitope
of HIV was inserted into the BamHI site using cassette

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insertion of ready-made oligonucleotides encoding the
HIV epitope. The required base substitutions at the
BamHI site did not lead to any amino acid changes in
the vector, although two amino acids (Asp and Glu)
changed places. This change did not have any
deleterious effect since in vitro made vector RNA
induced cell infection with wild type efficiency. Fig.
12 shows the sequences in the area of interest in the
epitope carrier. In preliminary experiments, it has
been shown that chimaeric proteins were produced. The
proteins can be immunoprecipitated with anti-HIV anti-
bodies. It is to be expected that these are also used
for production of chimaeric virus particles that can be
used for vaccine preparation against HIV. Such par-
ticles are shown in Fig. 12, lower part.

List of references

1)   Bishop, D.H.L. (1990). Gene expression using
        insect cells and viruses. In current Opinion in
        Biotechnology, Vol. 1, Rosenberg, M., and Moss,
        B., eds. (London: Current Opinion Ltd.), pp.
        62-67.

2)   Moss, B. (1990). Regulation of Vaccinia virus
        transcription. Ann. Rev. Biochem. 59, 661-688.

3)   Moss, B, and Flexner, C. (1989). Vaccinia virus
        expression vectors. Ann. N.Y. Acad Sci. 569,
        86-103.

4)   Garoff, H., Kondor-Koch, C., and Riedel, H.
        (1982). Structure and assembly of alphaviruses.
        Curr. Top. Microbiol. Immunol. 99, 1-50.

5)   Strauss, E.G., and Strauss, J.H. (1986).

Structure and replication of the alphavirus genome. In The Togaviridae and Flaviviridae, Vol. Schlesinger, S.S., and Schlesinger, M.J., eds (New york: Plenum Press), pp. 35-90.

6)   Garoff, H., Frischauf, A.-M., Simons, K., Lehrach, H, and Delius, H. (1980). Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. Nature 288, 236-241.

7)   Takkinen, K. (1986). Complete nucleotide sequence of the nonstructural protein genes of Semliki forest virus. Nucl. Acids Res. 14, 5667-5682.

8)   de Groot, R.J., Hardy, W.R., Shirako, Y., and Strauss, J.H. (1990). Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteinase. Evidence for temporal regulation of polyprotein processing in vivo. EMBO J. 9, 2631-2638.

9)   Hahn, Y.S., Strauss, E.G., and Strauss, J.H. (1989b). Mapping of RNA-temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. J. Virol. 63, 3142-3150.

10)   Mi, S., Durbin, R., Huang, H.V., Rice, C.M., and Stollar, V. (1989). Association of the Sindbis virus RNA methyltransferase activity with the nonstructural protein nsP1. Virology 170, 385-391.

11)   Ding, M., and Schlesinger, M.J. (1989). Evidence that Sindbis virus nsP2 is an auto-

42

protease which processes the virus non-
structural polyprotein. Virology 171, 280-284.

12)   Hardy, W.R., and Strauss, J.H. (1989). Proces-
         sing the nonstructural polyproteins of Sindbis
         virus: nonstructural proteinase is in the C-
         terminal half of nsP2 and functions both in cis
         and in trans. J.Virol. 63, 4653-4664.

13)   Li, G., La Starza, M.W., Hardy, W.R., Strauss,
         J.H., and Rice, C.M. (1990). Phosphorylation of
         Sindbis virus nsP3 in vivo and in vitro.

14)   Peränen, J., Takkinen, K., Kalkkinen, N., and
         Kääriäinen, L. (1988). Semliki Forest virus-
         specific nonstructural protein nsP3 is a phos-
         phoprotein. J. Gen. Virol. 69, 2165-2178.

15)   Hahn, Y.S., Grakoui, A., Rice, C.M., Strauss,
         E.G., and Strauss, J.H. (1989a). Mapping of
         RNA-temperature-sensitive mutants of Sindbis
         virus: complementation group F mutants have
         lesions in nsP4.

16)   Sawicki, D.L., Barkhimer, D.B. Sawicki, S.G.,
         Rice, C.M., and Schlesinger, S. (1990).
         Temperature sensitive shut-off of alphavirus
         minus strand RNA synthesis maps to a nonstruc-
         tural protein, nsP4. Virology 174, 43-52.

17)   Grakoui, A., Levis, R., Raju, R., Huang, H.V.,
         and Rice, C.M. (1989). A cis-acting mutation in
         the Sindbis virus junction region which affects
         subgenomic RNA synthesis. J. Virol. 63, 5216-
         5227.

18)   Levis, R., Schlesinger, S., and Huang, H.V.

(1990). Promoter for Sindbis virus RNA-
dependent subgenomic RNA transcription. J.
Virol. 64, 1726-1733.

19)   Schlesinger, S.S., and Schlesinger, M.J.
        (1986). Formation and assmebly of alphavirus
        glycoproteins. In The Togaviridae and Flavivi-
        ridae, Vol. Schlesinger, S.S., and Schlesinger,
        M.J., eds. (New York: Plenum Press), pp.121-
        148.

20)   Hahn, C.S., and Strauss, J.H. (1990). Site-
        directed mutagenesis of the proposed catalytic
        amino acids of the Sindbis virus capsid protein
        autoprotease. J. Virol. 64, 3069-3073.

21)   Melançon, P., and Garoff, H. (1987). Processing
        of the Semliki Forest virus structural poly-
        protein; Role of the capsid protease. J. Virol.
        61, 1301-1309.

22)   Bonatti, S., Migliaccio, G., Blobel, G., and
        Walter, P (1984). Role of the signal recogni-
        tion particle in the membrane assembly of Sind-
        bis viral gycoprotein. Eur. J. Biochem. 140,
        499-502.

23)   Garoff, H., Simons, K., and Dobberstein, B.
        (1978). Assembly of Semliki Forest virus
        membrane glycoproteins in the membrane of the
        endoplasmic reticulum in vitro. J. Mol. Biol.
        124, 587-600.

24)   Garoff, H., Huylebroeck, D., Robinson, A.,
        Tillman, U., and Liljeström, P. (1990). The
        signal sequence of the p62 protein of Semliki
        Forest virus is involved in initiation but not
        in completing chain translocation. J. Cell

44
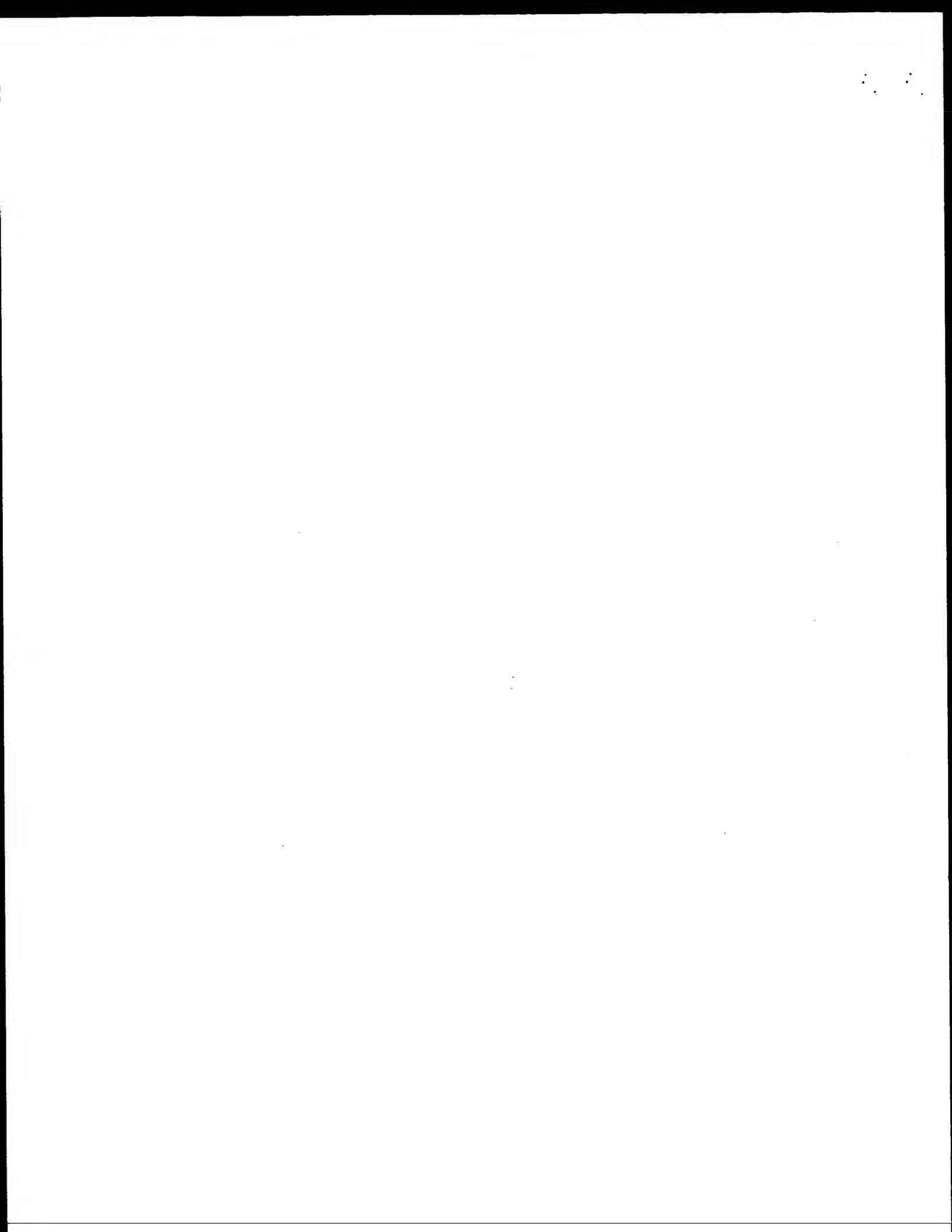
Biol. 111, 867-876.

25)　　　Melançon, P., and Garoff, H. (1986). Reinitia-
tion of translocation in the Semliki Forest
virus structural polyprotein: Identification of
the signal for the E1 glycoprotein. EMBO J. 5,
1551-1560.

26)　　　Lobigs, M., Zhao, H., and Garoff, H. (1990b).
Function of Semliki Forest virus E3 peptide in
virus assembly: Replacement of E3 with an arti-
ficial signal peptide abolishes spike heterodi-
merization and surface expression of E1. J.
Virol. 64, 4346-4355.

27)　　　de Curtis, I., and Simons, K. (1988). Dissec-
tion of Semliki Forest virus glycoprotein
delivery from the trans-Golgi network to the
cell surface in permeabilized BHK cells. Proc.
Natl. Acad. Sci. USA, 85, 8052-8056.

28)　　　Helenius, A., Kielian, M., Mellman, I., and
Schmid, S. (1989). Entry of enveloped viruses
into their host cells. In Cell Biology of Virus
Entry, Replication, and Pathogenesis, Vol. 90,
Compans, R. W., Helenius, A., and Oldstone,
M.B.A., eds. (New York: Alan R. Liss, Inc.),
pp. 145-161.

29)　　　Lobigs, M., and Garoff, H. (1990). Fusion
function of the Semliki Forest virus spike is
activated by proteolytic cleavage of the
envelope glycoprotein p62. J. Virol. 64, 1233-
1240.

30)　　　Lobigs, M., Wahlberg, J.M., and Garoff, H.
(1990a). Spike protein oligomerization control

of Semliki Forest virus fusion. J. Virol. 64, 5214-5218.

31)   Wahlberg, J.M., Boere, W.A., and Garoff, H. (1989). The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to mildly acidic pH during virus maturation. J. Virol. 63, 4991-4997.

32)   Ziemiecki, A., Garoff, H., and Simons, K. (1980). Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. J. Gen. Virol. 50, 111-123.

33)   Fuller, S.D. (1987). The T=4 envelope of Sindbis virus is organized by interactions with a complementary T=3 capsid. Cell 48, 923-934.

34)   Wengler, G. (1980). Effects of alphaviruses on host cell macromolecular synthesis. In The Togaviruses, Vol. Schlesinger, R. W., eds. (New York: Academic Press, Inc.), pp. 459-472.

35)   Stollar, V. (1980). Defective interfering alphaviruses. In The Togaviruses, Vol. Schlesinger, R.W., eds. (New York: Academic Press), pp. 427-457.

36)   Boere, W.A.M., Harmsen, T., Vinje, J., Benaissa-Trouw, B.J., Kraaijeeveld, C.A., and Snippe. H. (1984). Identification of distinct antigenic determinants on Semliki Forest virus by using monoclonal antibodies with different antiviral activities. J. Virol. 52, 575-582.

37)   Greiser-Wilke, I., Moennig, V., Kaaden, O.-R.,

and Figueiredo, L.T.M. (1989). Most alpha-viruses share a conserved epitopic region on their nucleocapsid protein. J. Gen. Virol. 70, 743-748.

38)   Kondor, K.C., Bravo, R., Fuller, S.D., Cutler, D., and Garoff, H. (1985). Exocytotic pathways exist to both the apical and the basolateral cell surface of the polarized epithelial cell MDCK. Cell 43, 297-306.

39)   Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning. A Laboratory Manual. (Cold Spring Harbor: Cold spring Harbor Laboratory Press).

40)   Benson, S.A. (1984). A rapid procedure for isolation of DNA fragments from agarose gels. Bio Techniques 2, 66-68.

41)   Silhavy, T.J., Berman, M.L., and Enquist, L.W. (1984). Experiments with Gene Fusions. (New York: Cold Spring Harbor Laboratory Press).

42)   Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103-119.

43)   Chung, C.T., and Miller, R.T. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucl. Acids Res. 16, 3580.

44)   Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific muta-

47

genesis without phenotypic selection. Meth.
Enzymol. 154, 367-382.

45)   Su, T.-Z., and El-Gewely, M.R. (1988). A :
multisite-directed mutagenesis using T7 DNA
polymerase: application for reconstructing a
mammalian gene. Gen 69, 81-89.

46)   Krieg, P.A., and Melton, D.A. (1987). In vitro
RNA synthesis with SP6 RNA polymerase. Meth.
Enzymol. 155, 397-415.

47)   Rice, C.M., Levis, R., Strauss, J.H., and
Huang, H.V. (1987). Production of infectious
RNA transcripts from Sindbis virus cDNA clones:
Mapping of lethal mutations, rescue of a
temperature-sensitive marker, and in vitro
mutagenesis to generate defined mutants. J.
Virol. 61, 3809-3819.

48)   Cutler, D.F., and Garoff, H. (1986). Mutants of
the membrane-binding region of Semliki Forest
virus E2 protein. I. Cell surface transport and
fusogenic activity. J. Cell Biol. 102, 889-901.

49)   Chamberlain, J.P. (1979). Fluorographic detec-
tion of radioactivity in polyacrylamide gels
with watersoluble fluor, sodium salicylate.
Anal. Biochem. 98, 132-135.

50)   Gubler, U., and Hoffman, B.J. (1983). A simple
and very efficient method for generating cDNA
libraries. Gene 25, 263-269.

51)   Haymerle, H., Herz, J., Bressan, G.M., Frank,
R., and Stanley, K.K. (1986). Efficient con-
struction of cDNA libraries in plasmid expres-

48

sion vectors using an adaptor strategy. Nucl.
Acids Res. 14, 8615-8124.

52)   Davis, N.L., Willis, L.V., Smith, J.F., and
Johnston, R.E. (1989). In vitro synthesis of
infectious Venezuelan Equine Encephalitis virus
RNA from a cDNA clone: Analysis of a viable
deletion mutant. Virology 171, 189-204.

53)   Niesters, H.G., and Strauss, J.H. (1990a).
Defined mutations in the 5' nontranslated
sequence of Sindbis virus RNA. J. Virol. 64,
4162-4168.

54)   Niesters, H.G.M., and Strauss, J.H. (1990b).
Mutagenesis of the conserved 51-nucleotide
region of Sindbis virus. J. Virol. 64, 1639-
1647.

55)   Tsiang, M., Weiss, B.G., and Schlesinger, S.
(1988). Effects of 5'-terminal modifications on
the biological activity of defective inter-
fering RNAs of Sindbis virus. J. Virol. 62, 47-
53.

56)   Kuhn, R.J., Hong, Z., and Strauss, J.H. (1990).
Mutagenesis of the 3' nontranslated region of
Sindbis virus RNA. J. Virol. 64, 1465-1476.

57)   Levis, R., Weiss, B.G., Tsiang, M., Huang, H.,
and Schlesinger, S. (1986). Deletion mapping of
Sindbis virus DI RNAs derived from cDNAs
defines the sequences essential for replication
and packaging. Cell 44, 137-145.

58)   Kozak, M. (1989). The scanning model for trans-
lation: an update. J. Cell Biol. 108, 229-241.

49

59) Weiss, B., Nitschko, H., Ghattas, I., Wright, R., and Schlesinger, S. (1989). Evidence for specificity in the encapsidation of Sindbis virus RNAs. J. Virol. 63, 5310-5318.

60) Davis NL, Pence DF, Meyer WJ, Schmaljohn AL and Johston RE (1987). Alternative forms of a strain-specific neutralizing antigenic site on the Sindbis virus E2 glycoprotein. Virology 161:101-108.

61) Mendoza QP, Stanley J and Griffin DE (1988). Monoclonal antibodies to the E1 and E2 glyco-proteins of Sindbis virus: Definition of epitopes and efficiency of protection from fatal encephalitis. J. Gen. Virol. 70:3015-3022.

62) Vrati S, Fernon CA, Dalgarno L and Weir RC (1988). Location of a major antigenic site involved in Ross River virus neutralization. Virology 162:346-353.

63) Grosfeld H, Velan B, Leitner M. Cohen S, Lustig S, Lachmi B and Shafferman A (1989). Semliki Forest virus E2 envelope epitopes induce a nonneutralizing humoral response which protects mice against lethal challenge. J. Virol. 63:3416-3422.

64) Zerial, M., Melançon, P., Schneider, C., and Garoff, H. (1986). The transmembrane segment of the human transferrin receptor functions as a signal peptide. EMBO J. 5, 1543-1550.

65) Schneider, C., Owen, M.J., Banville, D., and Williams, J.G. (1984). Primary structure of

human transferrin receptor deduced from the
mRNA sequence. Nature 311, 675-678.

66)   Ratner L, Haseltine W, Patarca R, Livak KJ,
        Starcich B, Josephs SF, Doran ER, Rafalki JA,
        Whitehorn EA, Baumeister K, Ivanoff L, Petteway
        SR, Pearson ML, Lautenberger JA, Papas TS,
        Ghrayeb J, Chang NT, Gallo RC and Wong-Staal F
        (1985). Complete nucleotide sequence of the
        AIDS virus, HTLVIII. Nature 313:277-284.

67)   AIDS (1988). Sci.Am. 259. A single-topic issue
        on HIV biology.

68)   Desrosiers RC, Wyand MS, Kodama T, Ringler DJ,
        Arthur LO, Sehgal PK, Letvin NL, King NW and
        Daniel MD (1989). Vaccine protection against
        simian immunodeficiency virus infection.

69)   Ginsberg H, Brown F, Lerner RA and Chanoch RM
        (1988). Vaccines 1988. New chemical and genetic
        approaches to vaccination, Cold Spring Harbor
        Laboratory, 396 pp.

70)   Burke KL, Dunn G, Ferguson M, Minor PD and
        Almond JW (1988). Antigen chimeras of polio-
        virus as potential new vaccines. Nature 332:81-
        82.

71)   Colbere-Garapin F, Christodoulou C, Crainic R,
        Garapin A-C and Candrea A (1988). Addition of a
        foreign oligopeptide to the major capsid
        protein of poliovirus. Proc. Natl. Acad. Sci.
        USA 85:8668-8672.

72)   Evans DJ, McKeating J, Meredith JM, Burke KL,
        Katrak K, John A, Ferguson M, Minor PD, Weiss

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RA and Almond JW (1989). An engineered polio-
virus chimaera elicits broadly reactive HIV-1
neutralizing antibodies. Nature 339:385-388.

Claims

1. An RNA molecule derived from an alphavirus RNA genome and capable of efficient infection of animal host cells, which RNA molecule comprises the complete alphavirus RNA genome regions, which are essential to replication of the said alphavirus RNA, and further comprises an exogenous RNA sequence capable of expressing its function in said host cell, said exogenous RNA sequence being inserted into a region of the RNA molecule which is non-essential to replication thereof.

2. The RNA of claim 1, wherein the said alphavirus is Semliki Forest virus (SFV).

3. The RNA of claim 1 or 2, wherein the exogenous RNA sequence encodes a protein, a polypeptide or a peptide sequence defining an exogenous antigenic epitope or determinant.

4. The RNA of claim 3 wherein the exogenous RNA sequence encodes an epitope sequence of a structural protein of an immunodeficiency virus inclusive of the human immunodeficiency virus (HIV) types.

5. The RNA of any preceding claim, wherein the alphavirus derived RNA molecule regions comprise a 5' terminal portion, the coding region(s) for non structural proteins required for RNA replication, the subgenome promoter region and a 3' terminal portion of said viral RNA.

6. The RNA of claim 2, 3 or 5, wherein the exogenous RNA sequence encodes a foreign polypeptide or protein and is integrated into the SFV subgenomic 26S RNA substituting deleted parts thereof.

7. The RNA of claim 2, 3, 4 or 5, wherein the exogenous RNA sequence encodes a foreign viral epitopic peptide sequence and is located in a region of the RNA coding for structural alphavirus proteins enabling the exogenous RNA to be expressed as said viral epitope as part of the matured virus particle.

8. The RNA of claim 2, 3, 4 or 5, wherein the exogenous RNA sequence encodes a foreign viral epitopic peptide sequence inserted into the p62 spike precursor subunit encoding region of the SFV genome.

9. An RNA expression vector comprising the RNA of any preceding claim packaged into infectious particles comprising the RNA within the alphavirus nucleocapsid and surrounded by membrane with alphavirus spike proteins.

10. The vector of claim 9, wherein the RNA has a total size corresponding to the wild type alphavirus RNA genome or deviating therefrom to an extent compatible with package of the RNA into the infectious particles.

11. DNA transcription vector comprising a cDNA having one strand complementary to the RNA of any of claims 1 to 8.

12. A DNA expression vector comprising a full-length or partial cDNA complementary to alphavirus RNA or parts thereof and located immediately downstream of the SP6 RNA polymerase promoter and having a 5'ATGG or 5'GATGG or any other 5' terminus and a $TTTCCA_{69}ACTAGT$ or any other 3' terminus.

13. The vector of claim 12 having portions of the viral cDNA deleted, the deletions comprising the complete or part of the region(s) encoding the virus structural proteins, and further comprising an integrated polylinker region, which may correspond to BamHI-SmaI-XmaI, inserted at a location which enables an exogenous DNA fragment encoding a foreign poly-peptide or protein to be inserted into the vector cDNA for subsequent expression in an animal host cell.

14. The vector of claim 12 or 13 wherein the alphavirus is SFV.

15. The vector of claim 12 or 14 comprising full-length cDNA and further comprising an exogenous DNA fragment encoding a foreign epitopic peptide sequence or antigenic determinant inserted into a region of the viral structural proteins.

16. The vector of claim 15 wherein the exogenous DNA fragment is inserted into the p62 spike precursor subunit encoding region of the SFV cDNA.

17. The vector of any preceding claim comprising an SFV derived cDNA which carries a conditionally lethal SFV mutation in the region encoding the p62 cleavage site, a cellularly uncleavable but extracellularly cleavable form of p62 being expressed.

18. The vector of claim 13 comprising SFV-derived cDNA, the vector being pSFV1, pSFV2 or pSFV3 having a structure as shown in Fig. 8.

19. An RNA transcript derived from transcription of the DNA-vector of any of claims 12-18 carrying an exogenous DNA fragment.

20. A method to produce the vector of claim 9 or 10

wherein the alphavirus derived RNA lacks part of or the
complete region(s) encoding the structural viral proteins, the
method comprising cotransfection of animal host cells with the
RNA transcript of claim 19, wherein the alphavirus RNA lacks
part(s) of or the complete region(s) encoding the viral struc-
tural proteins, with helper RNA transcribed in vitro from a
helper DNA vector and culturing the host cells.

21. The method of claim 20 wherein the cotransfection is
produced by electroporation of the host cells.

22. Helper vector for use in the method according to
claim 20 or 21, said vector being comprised of the DNA vector
of claim 12 wherein the regions encoding non structural virus
proteins are almost completely deleted, including sequences
encoding RNA signals for packaging of RNA into nucleocapsid
particles, but the 5' and 3' signals needed for RNA replication
and the region encoding the promoter for the structural sub-
genome are in addition to those encoding the structural region
preserved.

23. Helper vector of claim 22 wherein the cDNA has its
origin from SFV and the deletion extends from the AccI (308) to
the AccI (6399) restriction endonuclease site of the full-
length cDNA vector of claim 12.

24. Helper vector of claim 22 and 23 where the structural
region contains the mutation described in claim 17 or another
conditionally lethal mutation.

25. The method of claim 20 wherein cells transformed to
produce helper RNA according to claims 20, 22 or 23 are trans-
fected with RNA transcript of claim 19.

26. A host cell of animal origin transformed with the RNA
of any of claims 1-8, the DNA transcription vector of claims 11
or the DNA vector of any of claims 12-18 carrying an exogenous
DNA fragment.

27. The host cell of claim 26 wherein the cell is an
avian, a mammalian, a reptilian, an amphibian, an insecticidal
or a fish cell.

28. The host cell of claim 27 which is the hamster BHK
cell.

29. A method to produce the transformed host cell of
claim 26, 27 or 28 comprising transfection of the cell with the
RNA of any of claim 1-8, with the cDNA of claim 11 or of any of

claims 12-18 carrying an exogenous DNA fragment or infection of the cell with the infectious viral particles of claim 9 or 10.

30. The method of claim 29 wherein the transfection is produced by electroporation of the host cell.

31. A method for the production of a polypeptide or protein comprising infection of animal host cells with infectious particles according to claim 9 or 10, containing exogenous RNA encoding said polypeptide or protein and produced according to method of claim 20 or 21, culturing the said transformed cells to express the exogenous RNA and isolation and purification of the product formed by said expression.

32. A method for the production of a polypeptide or protein comprising in vitro transcription of the cDNA of the vector of any of claims 11-18 carrying an exogenous DNA fragment coding for the polypeptide or protein, transfection of animal host cells with the produced RNA transcript, transformed animal host cells being obtained harbouring the RNA transcript, culturing the said transformed cells to express the exogenous RNA and isolation and purification of the product formed by said expression.

33. The metod of claim 32 wherein the vector cDNA is comprised of the cDNA of the vector of claim 17 carrying the exogenous DNA fragment.

34. An antigen consisting of a chimaeric alphavirus having an exogenous epitopic peptide sequence or antigenic determinant inserted into its structural proteins.

35. The antigen of claim 34 wherein the chimaeric alphavirus is derived from SFV.

36. The antigen of claim 34 or 35, wherein the exogenous epitopic peptide sequence is comprised of an epitopic peptide sequence derived from a structural protein of a virus belonging to the immunodeficiency virus class inclusive of the human immunodeficiency virus types.

37. Vaccine preparation comprising the antigen of claim 34, 35 or 36 as immunizing component.

38. Vaccine of claim 37 wherein the chimaeric alphavirus is attenuated by comprising the conditionally lethal SFV mutation of claim 17, an amber (stop codon) a temperature sensitive mutation or other mutation in its genome.

39. A method for the production of an antigen of claim

34, 35 or 36 comprising

a) in vitro transcription of the cDNA of the vector of any of claims 11-18 carrying an exogenous DNA fragment encoding the foreign epitopic peptide sequence or antigenic determinant and transfection of animal host cells with the produced RNA transcript, or

b) transfection of animal host cells with the said cDNA of the above step a),

culturing the transfected cells and recovering the chimaeric alphavirus antigen.

40. The method of claim 32, 33 or 39 wherein the transfection is produced by electroporation of the host cell.

41. A method for the production of an antigen in an organism by using in vivo infection with infectious particles according to claim 9 or 10 containing exogenous RNA encoding an exogenous epitopic peptide sequence or antigenic determinant, and produced according the claim 20 or 21.
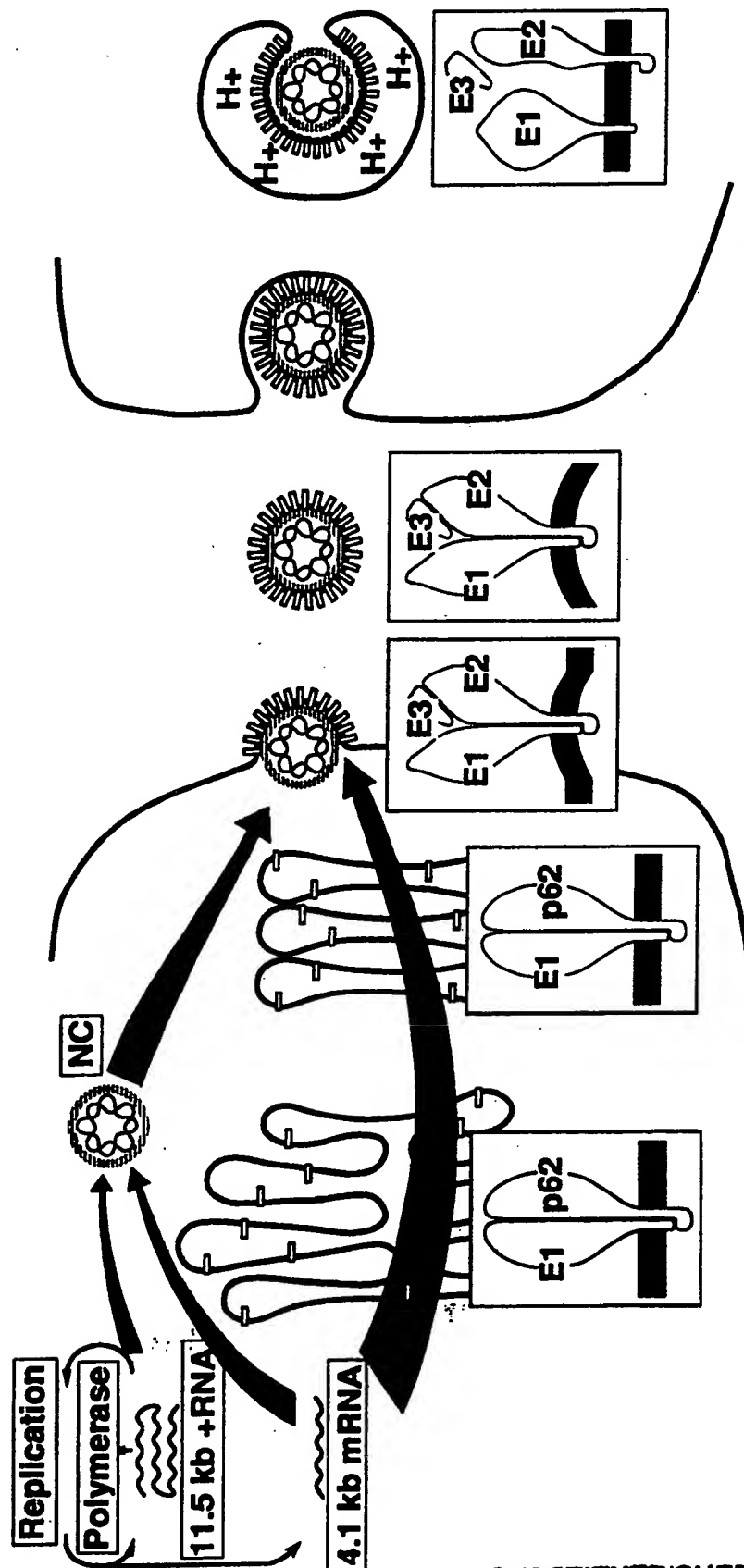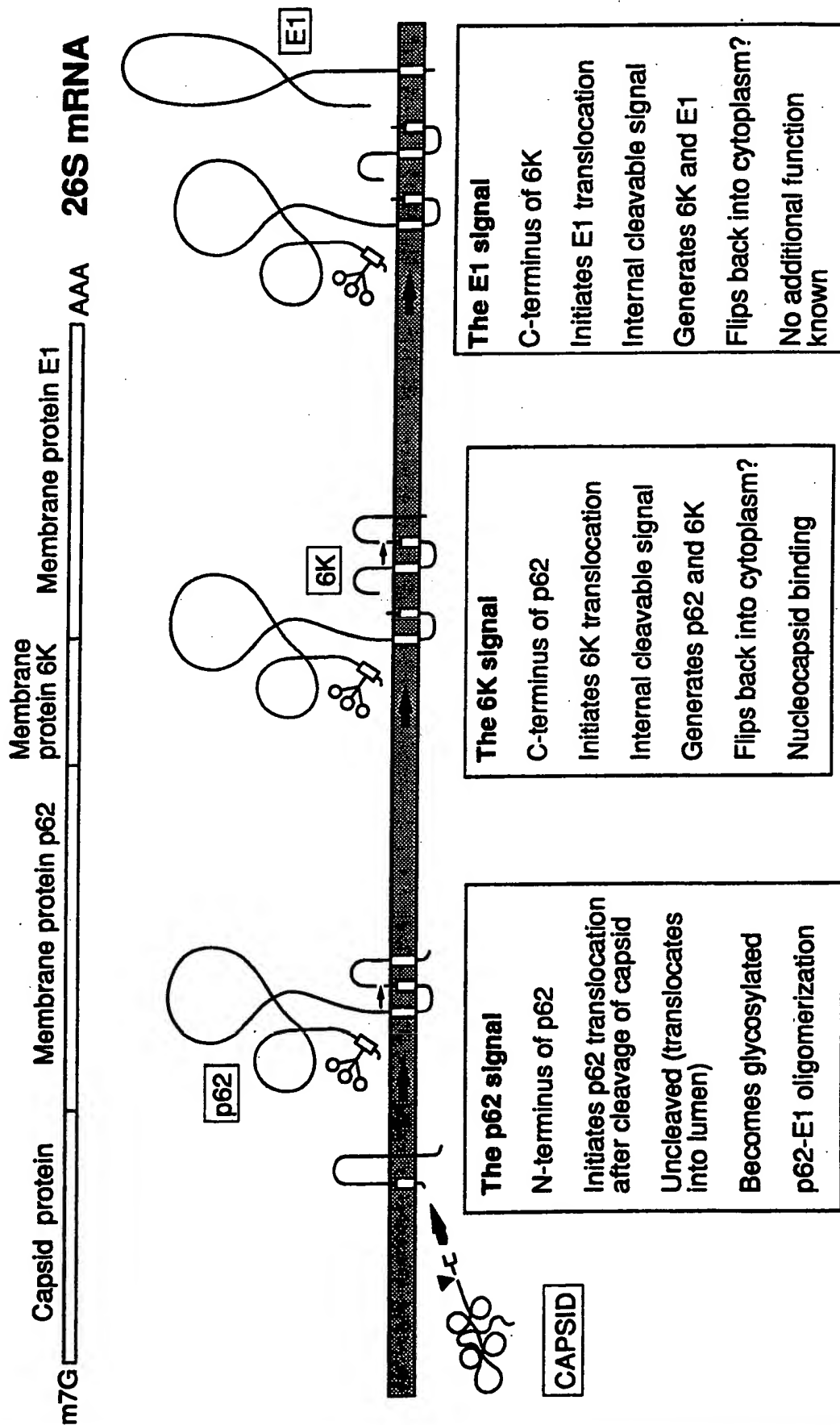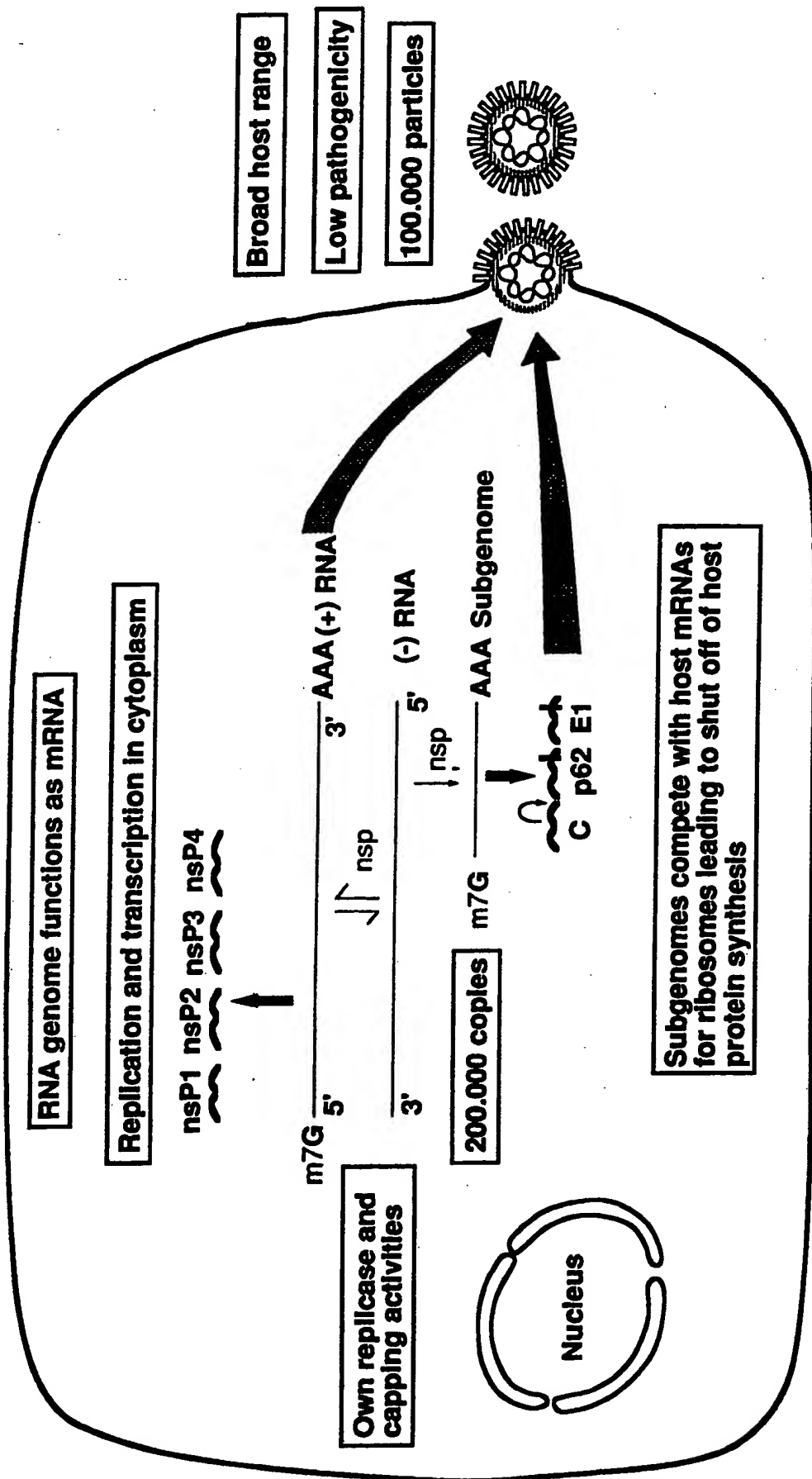

Figure 1

Figure 2
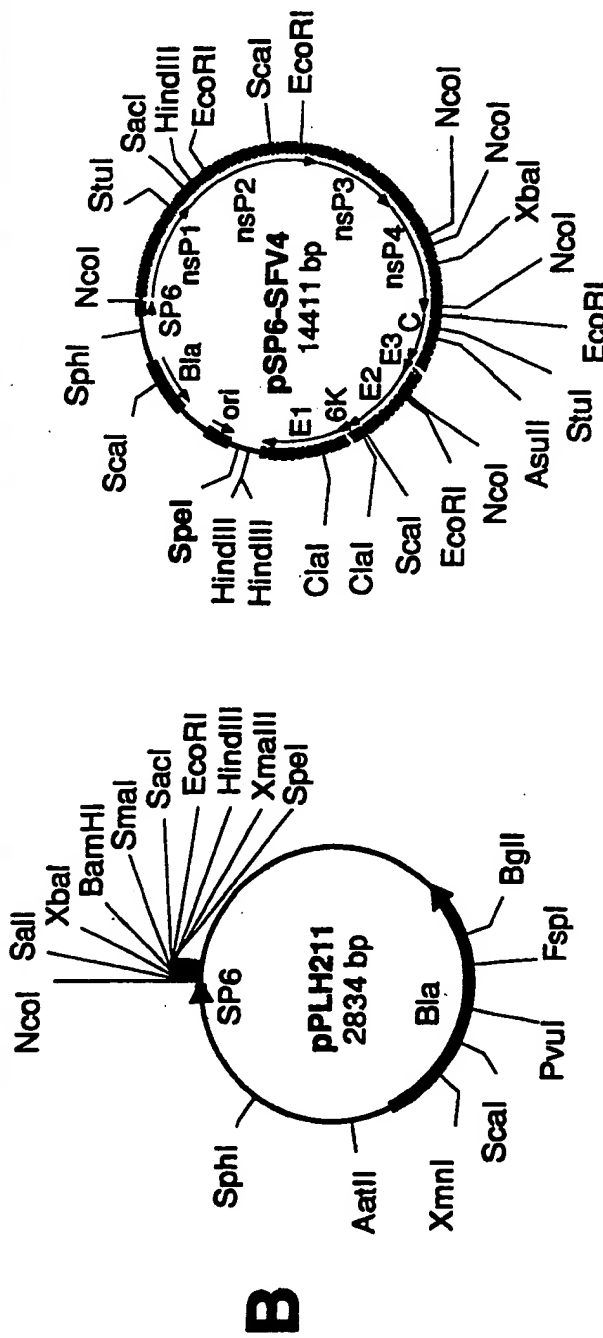
Figure 3

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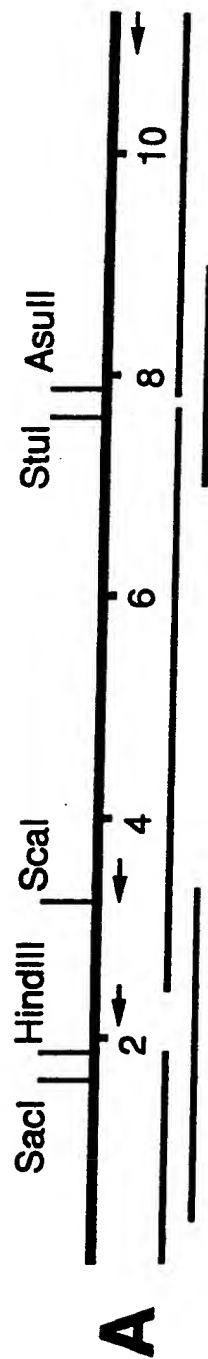
Figure 4

GATGGCGGAT GTGTGACATA CACGACGCCA AAAGATTTTG TTCCAGCTCC TGCCACCTCC 60

GCTACGCGAG AGATTAACCA CCCACG ATG GCC GCC AAA GTG CAT GTT GAT ATT 113
                             Met Ala Ala Lys Val His Val Asp Ile
                                                            5

GAG GCT GAC AGC CCA TTC ATC AAG TCT TTG CAG AAG GCA TTT CCG 158
Glu Ala Asp Ser Pro Phe Ile Lys Ser Leu Gln Lys Ala Phe Pro
10   15   20

TCG TTC GAG GTG GAG TCA TTG CAG GTC ACA CCA AAT GAC CAT GCA 203
Ser Phe Glu Val Glu Ser Leu Gln Val Thr Pro Asn Asp His Ala
25   30   35

AAT GCC AGA GCA TTT TCG CAC CTG GCT ACC AAA TTG ATC GAG CAG 248
Asn Ala Arg Ala Phe Ser His Leu Ala Thr Lys Leu Ile Glu Gln
40   45   50

GAG ACT GAC AAA GAC ACA CTC ATC TTG GAT ATC GGC AGT GCG CCT 293
Glu Thr Asp Lys Asp Thr Leu Ile Leu Asp Ile Gly Ser Ala Pro
55   60   65

TCC AGG AGA ATG ATG TCT ACG CAC AAA TAC CAC TGC GTA TGC CCT 338
Ser Arg Arg Met Met Ser Thr His Lys Tyr His Cys Val Cys Pro
70   75   80

ATG CGC AGC GCA GAA GAC CCC GAA AGG CTC GAT AGC TAC GCA AAG 383
Met Arg Ser Ala Glu Asp Pro Glu Arg Leu Asp Ser Tyr Ala Lys
85   90   95

AAA CTG GCA GCG GCC TCC GGG AAG GTG CTG GAT AGA GAG ATC GCA 428
Lys Leu Ala Ala Ala Ser Gly Lys Val Leu Asp Arg Glu Ile Ala
100   105   110

GGA AAA ATC ACC GAC CTG CAG ACC GTC ATG GCT ACG CCA GAC GCT 473
Gly Lys Ile Thr Asp Leu Gln Thr Val Met Ala Thr Pro Asp Ala
115   120   125

GAA TCT CCT ACC TTT TGC CTG CAT ACA GAC GTC ACG TGT CGT ACG 518
Glu Ser Pro Thr Phe Cys Leu His Thr Asp Val Thr Cys Arg Thr
130   135   140

GCA GCC GAA GTG GCC GTA TAC CAG GAC GTG TAT GCT GTA CAT GCA 563
Ala Ala Glu Val Ala Val Tyr Gln Asp Val Tyr Ala Val His Ala
145   150   155

CCA ACA TCG CTG TAC CAT CAG GCG ATG AAA GGT GTC AGA ACG GCG 608
Pro Thr Ser Leu Tyr His Gln Ala Met Lys Gly Val Arg Thr Ala
160   165   170

TAT TGG ATT GGG TTT GAC ACC ACC CCG TTT ATG TTT GAC GCG CTA 653
Tyr Trp Ile Gly Phe Asp Thr Thr Pro Phe Met Phe Asp Ala Leu
175   180   185

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Figure 5 (2)   6/33

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GCA GGC GCG TAT CCA ACC TAC GCC ACA AAC TGG GCC GAC GAG CAG  698
Ala Gly Ala Tyr Pro Thr Tyr Ala Thr Asn Trp Ala Asp Glu Gln
190             195             200

GTG TTA CAG GCC AGG AAC ATA GGA CTG TGT GCA GCA TCC TTG ACT  743
Val Leu Gln Ala Arg Asn Ile Gly Leu Cys Ala Ala Ser Leu Thr
205             210             215

GAG GGA AGA CTC GGC AAA CTG TCC ATT CTC CGC AAG AAG CAA TTG  788
Glu Gly Arg Leu Gly Lys Leu Ser Ile Leu Arg Lys Lys Gln Leu
220             225             230

AAA CCT TGC GAC ACA GTC ATG TTC TCG GTA GGA TCT ACA TTG TAC  833
Lys Pro Cys Asp Thr Val Met Phe Ser Val Gly Ser Thr Leu Tyr
235             240             245

ACT GAG AGC AGA AAG CTA CTG AGG AGC TGG CAC TTA CCC TCC GTA  878
Thr Glu Ser Arg Lys Leu Leu Arg Ser Trp His Leu Pro Ser Val
250             255             260

TTC CAC CTG AAA GGT AAA CAA TCC TTT ACC TGT AGG TGC GAT ACC  923
Phe His Leu Lys Gly Lys Gln Ser Phe Thr Cys Arg Cys Asp Thr
265             270             275

ATC GTA TCA TGT GAA GGG TAC GTA GTT AAG AAA ATC ACT ATG TGC  968
Ile Val Ser Cys Glu Gly Tyr Val Val Lys Lys Ile Thr Met Cys
280             285             290

CCC GGC CTG TAC GGT AAA ACG GTA GGG TAC GCC GTG ACG TAT CAC  1013
Pro Gly Leu Tyr Gly Lys Thr Val Gly Tyr Ala Val Thr Tyr His
295             300             305

GCG GAG GGA TTC CTA GTG TGC AAG ACC ACA GAC ACT GTC AAA GGA  1058
Ala Glu Gly Phe Leu Val Cys Lys Thr Thr Asp Thr Val Lys Gly
310             315             320

GAA AGA GTC TCA TTC CCT GTA TGC ACC TAC GTC CCC TCA ACC ATC  1103
Glu Arg Val Ser Phe Pro Val Cys Thr Tyr Val Pro Ser Thr Ile
325             330             335

TGT GAT CAA ATG ACT GGC ATA CTA GCG ACC GAC GTC ACA CCG GAG  1148
Cys Asp Gln Met Thr Gly Ile Leu Ala Thr Asp Val Thr Pro Glu
340             345             350

GAC GCA CAG AAG TTG TTA GTG GGA TTG AAT CAG AGG ATA GTT GTG  1193
Asp Ala Gln Lys Leu Leu Val Gly Leu Asn Gln Arg Ile Val Val
355             360             365

AAC GGA AGA ACA CAG CGA AAC ACT AAC ACG ATG AAG AAC TAT CTG  1238
Asn Gly Arg Thr Gln Arg Asn Thr Asn Thr Met Lys Asn Tyr Leu
370             375             380

CTT CCG ATT GTG GCC GTC GCA TTT AGC AAG TGG GCG AGG GAA TAC  1283
Leu Pro Ile Val Ala Val Ala Phe Ser Lys Trp Ala Arg Glu Tyr
385             390             395
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AAG GCA GAC CTT GAT GAT GAA AAA CCT CTG GGT GTC CGA GAG AGG   1328
Lys Ala Asp Leu Asp Asp Glu Lys Pro Leu Gly Val Arg Glu Arg
400             405             410

TCA CTT ACT TGC TGC TGC TTG TGG GCA TTT AAA ACG AGG AAG ATG   1373
Ser Leu Thr Cys Cys Cys Leu Trp Ala Phe Lys Thr Arg Lys Met
415             420             425

CAC ACC ATG TAC AAG AAA CCA GAC ACC CAG ACA ATA GTG AAG GTG   1418
His Thr Met Tyr Lys Lys Pro Asp Thr Gln Thr Ile Val Lys Val
430             435             440

CCT TCA GAG TTT AAC TCG TTC GTC ATC CCG AGC CTA TGG TCT ACA   1463
Pro Ser Glu Phe Asn Ser Phe Val Ile Pro Ser Leu Trp Ser Thr
445             450             455

GGC CTC GCA ATC CCA GTC AGA TCA CGC ATT AAG ATG CTT TTG GCC   1508
Gly Leu Ala Ile Pro Val Arg Ser Arg Ile Lys Met Leu Leu Ala
460             465             470

AAG AAG ACC AAG CGA GAG TTA ATA CCT GTT CTC GAC GCG TCG TCA   1553
Lys Lys Thr Lys Arg Glu Leu Ile Pro Val Leu Asp Ala Ser Ser
475             480             485

GCC AGG GAT GCT GAA CAA GAG GAG AAG GAG AGG TTG GAG GCC GAG   1598
Ala Arg Asp Ala Glu Gln Glu Glu Lys Glu Arg Leu Glu Ala Glu
490             495             500

CTG ACT AGA GAA GCC TTA CCA CCC CTC GTC CCC ATC GCG CCG GCG   1643
Leu Thr Arg Glu Ala Leu Pro Pro Leu Val Pro Ile Ala Pro Ala
505             510             515

GAG ACG GGA GTC GTC GAC GTC GAC GTT GAA GAA CTA GAG TAT CAC   1688
Glu Thr Gly Val Val Asp Val Asp Val Glu Glu Leu Glu Tyr His
520             525             530

GCA GGT GCA GGG GTC GTG GAA ACA CCT CGC AGC GCG TTG AAA GTC   1733
Ala Gly Ala Gly Val Val Glu Thr Pro Arg Ser Ala Leu Lys Val
535             540             545

ACC GCA CAG CCG AAC GAC GTA CTA CTA GGA AAT TAC GTA GTT CTG   1778
Thr Ala Gln Pro Asn Asp Val Leu Leu Gly Asn Tyr Val Val Leu
550             555             560

TCC CCG CAG ACC GTG CTC AAG AGC TCC AAG TTG GCC CCC GTG CAC   1823
Ser Pro Gln Thr Val Leu Lys Ser Ser Lys Leu Ala Pro Val His
565             570             575

CCT CTA GCA GAG CAG GTG AAA ATA ATA ACA CAT AAC GGG AGG GCC   1868
Pro Leu Ala Glu Gln Val Lys Ile Ile Thr His Asn Gly Arg Ala
580             585             590

GGC GGT TAC CAG GTC GAC GGA TAT GAC GGC AGG GTC CTA CTA CCA   1913
Gly Gly Tyr Gln Val Asp Gly Tyr Asp Gly Arg Val Leu Leu Pro
595             600             605
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TGT GGA TCG GCC ATT CCG GTC CCT GAG TTT CAA GCT TTG AGC GAG   1958
Cys Gly Ser Ala Ile Pro Val Pro Glu Phe Gln Ala Leu Ser Glu
610             615             620

AGC GCC ACT ATG GTG TAC AAC GAA AGG GAG TTC GTC AAC AGG AAA   2003
Ser Ala Thr Met Val Tyr Asn Glu Arg Glu Phe Val Asn Arg Lys
625             630             635

CTA TAC CAT ATT GCC GTT CAC GGA CCG TCG CTG AAC ACC GAC GAG   2048
Leu Tyr His Ile Ala Val His Gly Pro Ser Leu Asn Thr Asp Glu
640             645             650

GAG AAC TAC GAG AAA GTC AGA GCT GAA AGA ACT GAC GCC GAG TAC   2093
Glu Asn Tyr Glu Lys Val Arg Ala Glu Arg Thr Asp Ala Glu Tyr
655             660             665

GTG TTC GAC GTA GAT AAA AAA TGC TGC GTC AAG AGA GAG GAA GCG   2138
Val Phe Asp Val Asp Lys Lys Cys Cys Val Lys Arg Glu Glu Ala
670             675             680

TCG GGT TTG GTG TTG GTG GGA GAG CTA ACC AAC CCC CCG TTC CAT   2183
Ser Gly Leu Val Leu Val Gly Glu Leu Thr Asn Pro Pro Phe His
685             690             695

GAA TTC GCC TAC GAA GGG CTG AAG ATC AGG CCG TCG GCA CCA TAT   2228
Glu Phe Ala Tyr Glu Gly Leu Lys Ile Arg Pro Ser Ala Pro Tyr
700             705             710

AAG ACT ACA GTA GTA GGA GTC TTT GGG GTT CCG GGA TCA GGC AAG   2273
Lys Thr Thr Val Val Gly Val Phe Gly Val Pro Gly Ser Gly Lys
715             720             725

TCT GCT ATT ATT AAG AGC CTC GTG ACC AAA CAC GAT CTG GTC ACC   2318
Ser Ala Ile Ile Lys Ser Leu Val Thr Lys His Asp Leu Val Thr
730             735             740

AGC GGC AAG AAG GAG AAC TGC CAG GAA ATA GTT AAC GAC GTG AAG   2363
Ser Gly Lys Lys Glu Asn Cys Gln Glu Ile Val Asn Asp Val Lys
745             750             755

AAG CAC CGC GGG AAG GGG ACA AGT AGG GAA AAC AGT GAC TCC ATC   2408
Lys His Arg Gly Lys Gly Thr Ser Arg Glu Asn Ser Asp Ser Ile
760             765             770

CTG CTA AAC GGG TGT CGT CGT GCC GTG GAC ATC CTA TAT GTG GAC   2453
Leu Leu Asn Gly Cys Arg Arg Ala Val Asp Ile Leu Tyr Val Asp
775             780             785

GAG GCT TTC GCT TGC CAT TCC GGT ACT CTG CTG GCC CTA ATT GCT   2498
Glu Ala Phe Ala Cys His Ser Gly Thr Leu Leu Ala Leu Ile Ala
790             795             800

CTT GTT AAA CCT CGG AGC AAA GTG GTG TTA TGC GGA GAC CCC AAG   2543
Leu Val Lys Pro Arg Ser Lys Val Val Leu Cys Gly Asp Pro Lys
805             810             815
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CAA TGC GGA TTC TTC AAT ATG ATG CAG CTT AAG GTG AAC TTC AAC   2588
Gln Cys Gly Phe Phe Asn Met Met Gln Leu Lys Val Asn Phe Asn
820   825   830

CAC AAC ATC TGC ACT GAA GTA TGT CAT AAA AGT ATA TCC AGA CGT   2633
His Asn Ile Cys Thr Glu Val Cys His Lys Ser Ile Ser Arg Arg
835   840   845

TGC ACG CGT CCA GTC ACG GCC ATC GTG TCT ACG TTG CAC TAC GGA   2678
Cys Thr Arg Pro Val Thr Ala Ile Val Ser Thr Leu His Tyr Gly
850   855   860

GGC AAG ATG CGC ACG ACC AAC CCG TGC AAC AAA CCC ATA ATC ATA   2723
Gly Lys Met Arg Thr Thr Asn Pro Cys Asn Lys Pro Ile Ile Ile
865   870   875

GAC ACC ACA GGA CAG ACC AAG CCC AAG CCA GGA GAC ATC GTG TTA   2768
Asp Thr Thr Gly Gln Thr Lys Pro Lys Pro Gly Asp Ile Val Leu
880   885   890

ACA TGC TTC CGA GGC TGG GCA AAG CAG CTG CAG TTG GAC TAC CGT   2813
Thr Cys Phe Arg Gly Trp Ala Lys Gln Leu Gln Leu Asp Tyr Arg
895   900   905

GGA CAC GAA GTC ATG ACA GCA GCA GCA TCT CAG GGC CTC ACC CGC   2858
Gly His Glu Val Met Thr Ala Ala Ala Ser Gln Gly Leu Thr Arg
910   915   920

AAA GGG GTA TAC GCC GTA AGG CAG AAG GTG AAT GAA AAT CCC TTG   2903
Lys Gly Val Tyr Ala Val Arg Gln Lys Val Asn Glu Asn Pro Leu
925   930   935

TAT GCC CCT GCG TCG GAG CAC GTG AAT GTA CTG CTG ACG CGC ACT   2948
Tyr Ala Pro Ala Ser Glu His Val Asn Val Leu Leu Thr Arg Thr
940   945   950

GAG GAT AGG CTG GTG TGG AAA ACG CTG GCC GGC GAT CCC TGG ATT   2993
Glu Asp Arg Leu Val Trp Lys Thr Leu Ala Gly Asp Pro Trp Ile
955   960   965

AAG GTC CTA TCA AAC ATT CCA CAG GGT AAC TTT ACG GCC ACA TTG   3038
Lys Val Leu Ser Asn Ile Pro Gln Gly Asn Phe Thr Ala Thr Leu
970   975   980

GAA GAA TGG CAA GAA GAA CAC GAC AAA ATA ATG AAG GTG ATT GAA   3083
Glu Glu Trp Gln Glu Glu His Asp Lys Ile Met Lys Val Ile Glu
985   990   995

GGA CCG GCT GCG CCT GTG GAC GCG TTC CAG AAC AAA GCG AAC GTG   3128
Gly Pro Ala Ala Pro Val Asp Ala Phe Gln Asn Lys Ala Asn Val
1000   1005   1010

TGT TGG GCG AAA AGC CTG GTG CCT GTC CTG GAC ACT GCC GGA ATC   3173
Cys Trp Ala Lys Ser Leu Val Pro Val Leu Asp Thr Ala Gly Ile
1015   1020   1025

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AGA TTG ACA GCA GAG GAG TGG AGC ACC ATA ATT ACA GCA TTT AAG    3218
Arg Leu Thr Ala Glu Glu Trp Ser Thr Ile Ile Thr Ala Phe Lys
1030            1035                1040

GAG GAC AGA GCT TAC TCT CCA GTG GTG GCC TTG AAT GAA ATT TGC    3263
Glu Asp Arg Ala Tyr Ser Pro Val Val Ala Leu Asn Glu Ile Cys
1045            1050                1055

ACC AAG TAC TAT GGA GTT GAC CTG GAC AGT GGC CTG TTT TCT GCC    3308
Thr Lys Tyr Tyr Gly Val Asp Leu Asp Ser Gly Leu Phe Ser Ala
1060            1065                1070

CCG AAG GTG TCC CTG TAT TAC GAG AAC AAC CAC TGG GAT AAC AGA    3353
Pro Lys Val Ser Leu Tyr Tyr Glu Asn Asn His Trp Asp Asn Arg
1075            1080                1085

CCT GGT GGA AGG ATG TAT GGA TTC AAT GCC GCA ACA GCT GCC AGG    3398
Pro Gly Gly Arg Met Tyr Gly Phe Asn Ala Ala Thr Ala Ala Arg
1090            1095                1100

CTG GAA GCT AGA CAT ACC TTC CTG AAG GGG CAG TGG CAT ACG GGC    3443
Leu Glu Ala Arg His Thr Phe Leu Lys Gly Gln Trp His Thr Gly
1105            1110                1115

AAG CAG GCA GTT ATC GCA GAA AGA AAA ATC CAA CCG CTT TCT GTG    3488
Lys Gln Ala Val Ile Ala Glu Arg Lys Ile Gln Pro Leu Ser Val
1120            1125                1130

CTG GAC AAT GTA ATT CCT ATC AAC CGC AGG CTG CCG CAC GCC CTG    3533
Leu Asp Asn Val Ile Pro Ile Asn Arg Arg Leu Pro His Ala Leu
1135            1140                1145

GTG GCT GAG TAC AAG ACG GTT AAA GGC AGT AGG GTT GAG TGG CTG    3578
Val Ala Glu Tyr Lys Thr Val Lys Gly Ser Arg Val Glu Trp Leu
1150            1155                1160

GTC AAT AAA GTA AGA GGG TAC CAC GTC CTG CTG GTG AGT GAG TAC    3623
Val Asn Lys Val Arg Gly Tyr His Val Leu Leu Val Ser Glu Tyr
1165            1170                1175

AAC CTG GCT TTG CCT CGA CGC AGG GTC ACT TGG TTG TCA CCG CTG    3668
Asn Leu Ala Leu Pro Arg Arg Arg Val Thr Trp Leu Ser Pro Leu
1180            1185                1190

AAT GTC ACA GGC GCC GAT AGG TGC TAC GAC CTA AGT TTA GGA CTG    3713
Asn Val Thr Gly Ala Asp Arg Cys Tyr Asp Leu Ser Leu Gly Leu
1195            1200                1205

CCG GCT GAC GCC GGC AGG TTC GAC TTG GTC TTT GTG AAC ATT CAC    3758
Pro Ala Asp Ala Gly Arg Phe Asp Leu Val Phe Val Asn Ile His
1210            1215                1220

ACG GAA TTC AGA ATC CAC CAC TAC CAG CAG TGT GTC GAC CAC GCC    3803
Thr Glu Phe Arg Ile His His Tyr Gln Gln Cys Val Asp His Ala
1225            1230                1235
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Figure 5 (7)   11/33

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ATG AAG CTG CAG ATG CTT GGG GGA GAT GCG CTA CGA CTG CTA AAA   3848
Met Lys Leu Gln Met Leu Gly Gly Asp Ala Leu Arg Leu Leu Lys
1240            1245            1250

CCC GGC GGC ATC TTG ATG AGA GCT TAC GGA TAC GCC GAT AAA ATC   3893
Pro Gly Gly Ile Leu Met Arg Ala Tyr Gly Tyr Ala Asp Lys Ile
1255            1260            1265

AGC GAA GCC GTT GTT TCC TCC TTA AGC AGA AAG TTC TCG TCT GCA   3938
Ser Glu Ala Val Val Ser Ser Leu Ser Arg Lys Phe Ser Ser Ala
1270            1275            1280

AGA GTG TTG CGC CCG GAT TGT GTC ACC AGC AAT ACA GAA GTG TTC   3983
Arg Val Leu Arg Pro Asp Cys Val Thr Ser Asn Thr Glu Val Phe
1285            1290            1295

TTG CTG TTC TCC AAC TTT GAC AAC GGA AAG AGA CCC TCT ACG CTA   4028
Leu Leu Phe Ser Asn Phe Asp Asn Gly Lys Arg Pro Ser Thr Leu
1300            1305            1310

CAC CAG ATG AAT ACC AAG CTG AGT GCC GTG TAT GCC GGA GAA GCC   4073
His Gln Met Asn Thr Lys Leu Ser Ala Val Tyr Ala Gly Glu Ala
1315            1320            1325

ATG CAC ACG GCC GGG TGT GCA CCA TCC TAC AGA GTT AAG AGA GCA   4118
Met His Thr Ala Gly Cys Ala Pro Ser Tyr Arg Val Lys Arg Ala
1330            1335            1340

GAC ATA GCC ACG TGC ACA GAA GCG GCT GTG GTT AAC GCA GCT AAC   4163
Asp Ile Ala Thr Cys Thr Glu Ala Ala Val Val Asn Ala Ala Asn
1345            1350            1355

GCC CGT GGA ACT GTA GGG GAT GGC GTA TGC AGG GCC GTG GCG AAG   4208
Ala Arg Gly Thr Val Gly Asp Gly Val Cys Arg Ala Val Ala Lys
1360            1365            1370

AAA TGG CCG TCA GCC TTT AAG GGA GCA GCA ACA CCA GTG GGC ACA   4253
Lys Trp Pro Ser Ala Phe Lys Gly Ala Ala Thr Pro Val Gly Thr
1375            1380            1385

ATT AAA ACA GTC ATG TGC GGC TCG TAC CCC GTC ATC CAC GCT GTA   4298
Ile Lys Thr Val Met Cys Gly Ser Tyr Pro Val Ile His Ala Val
1390            1395            1400

GCG CCT AAT TTC TCT GCC ACG ACT GAA GCG GAA GGG GAC CGC GAA   4343
Ala Pro Asn Phe Ser Ala Thr Thr Glu Ala Glu Gly Asp Arg Glu
1405            1410            1415

TTG GCC GCT GTC TAC CGG GCA GTG GCC GCC GAA GTA AAC AGA CTG   4388
Leu Ala Ala Val Tyr Arg Ala Val Ala Ala Glu Val Asn Arg Leu
1420            1425            1430

TCA CTG AGC AGC GTA GCC ATC CCG CTG CTG TCC ACA GGA GTG TTC   4433
Ser Leu Ser Ser Val Ala Ile Pro Leu Leu Ser Thr Gly Val Phe
1435            1440            1445
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AGC GGC GGA AGA GAT AGG CTG CAG CAA TCC CTC AAC CAT CTA TTC   4478
Ser Gly Gly Arg Asp Arg Leu Gln Gln Ser Leu Asn His Leu Phe
1450              1455              1460

ACA GCA ATG GAC GCC ACG GAC GCT GAC GTG ACC ATC TAC TGC AGA   4523
Thr Ala Met Asp Ala Thr Asp Ala Asp Val Thr Ile Tyr Cys Arg
1465              1470              1475

GAC AAA AGT TGG GAG AAG AAA ATC CAG GAA GCC ATT GAC ATG AGG   4568
Asp Lys Ser Trp Glu Lys Lys Ile Gln Glu Ala Ile Asp Met Arg
1480              1485              1490

ACG GCT GTG GAG TTG CTC AAT GAT GAC GTG GAG CTG ACC ACA GAC   4613
Thr Ala Val Glu Leu Leu Asn Asp Asp Val Glu Leu Thr Thr Asp
1495              1500              1505

TTG GTG AGA GTG CAC CCG GAC AGC AGC CTG GTG GGT CGT AAG GGC   4658
Leu Val Arg Val His Pro Asp Ser Ser Leu Val Gly Arg Lys Gly
1510              1515              1520

TAC AGT ACC ACT GAC GGG TCG CTG TAC TCG TAC TTT GAA GGT ACG   4703
Tyr Ser Thr Thr Asp Gly Ser Leu Tyr Ser Tyr Phe Glu Gly Thr
1525              1530              1535

AAA TTC AAC CAG GCT GCT ATT GAT ATG GCA GAG ATA CTG ACG TTG   4748
Lys Phe Asn Gln Ala Ala Ile Asp Met Ala Glu Ile Leu Thr Leu
1540              1545              1550

TGG CCC AGA CTG CAA GAG GCA AAC GAA CAG ATA TGC CTA TAC GCG   4793
Trp Pro Arg Leu Gln Glu Ala Asn Glu Gln Ile Cys Leu Tyr Ala
1555              1560              1565

CTG GGC GAA ACA ATG GAC AAC ATC AGA TCC AAA TGT CCG GTG AAC   4838
Leu Gly Glu Thr Met Asp Asn Ile Arg Ser Lys Cys Pro Val Asn
1570              1757              1580

GAT TCC GAT TCA TCA ACA CCT CCC AGG ACA GTG CCC TGC CTG TGC   4883
Asp Ser Asp Ser Ser Thr Pro Pro Arg Thr Val Pro Cys Leu Cys
1585              1590              1595

CGC TAC GCA ATG ACA GCA GAA CGG ATC GCC CGC CTT AGG TCA CAC   4928
Arg Tyr Ala Met Thr Ala Glu Arg Ile Ala Arg Leu Arg Ser His
1600              1605              1610

CAA GTT AAA AGC ATG GTG GTT TGC TCA TCT TTT CCC CTC CCG AAA   4973
Gln Val Lys Ser Met Val Val Cys Ser Ser Phe Pro Leu Pro Lys
1615              1620              1625

TAC CAT GTA GAT GGG GTG CAG AAG GTA AAG TGC GAG AAG GTT CTC   5018
Tyr His Val Asp Gly Val Gln Lys Val Lys Cys Glu Lys Val Leu
1630              1635              1640

CTG TTC GAC CCG ACG GTA CCT TCA GTG GTT AGT CCG CGG AAG TAT   5063
Leu Phe Asp Pro Thr Val Pro Ser Val Val Ser Pro Arg Lys Tyr
1645              1650              1655
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SUBSTITUTE SHEET

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GCC GCA TCT ACG ACG GAC CAC TCA GAT CGG TCG TTA CGA GGG TTT   5108
Ala Ala Ser Thr Thr Asp His Ser Asp Arg Ser Leu Arg Gly Phe
1660            1665            1670

GAC TTG GAC TGG ACC ACC GAC TCG TCT TCC ACT GCC AGC GAT ACC   5153
Asp Leu Asp Trp Thr Thr Asp Ser Ser Ser Thr Ala Ser Asp Thr
1675            1680            1685

ATG TCG CTA CCC AGT TTG CAG TCG TGT GAC ATC GAC TCG ATC TAC   5198
Met Ser Leu Pro Ser Leu Gln Ser Cys Asp Ile Asp Ser Ile Tyr
1690            1695            1700

GAG CCA ATG GCT CCC ATA GTA GTG ACG GCT GAC GTA CAC CCT GAA   5243
Glu Pro Met Ala Pro Ile Val Val Thr Ala Asp Val His Pro Glu
1705            1710            1715

CCC GCA GGC ATC GCG GAC CTG GCG GCA GAT GTG CAC CCT GAA CCC   5288
Pro Ala Gly Ile Ala Asp Leu Ala Ala Asp Val His Pro Glu Pro
1720            1725            1730

GCA GAC CAT GTG GAC CTC GAG AAC CCG ATT CCT CCA CCG CGC CCG   5333
Ala Asp His Val Asp Leu Glu Asn Pro Ile Pro Pro Pro Arg Pro
1735            1740            1745

AAG AGA GCT GCA TAC CTT GCC TCC CGC GCG GCG GAG CGA CCG GTG   5378
Lys Arg Ala Ala Tyr Leu Ala Ser Arg Ala Ala Glu Arg Pro Val
1750            1755            1760

CCG GCG CCG AGA AAG CCG ACG CCT GCC CCA AGG ACT GCG TTT AGG   5423
Pro Ala Pro Arg Lys Pro Thr Pro Ala Pro Arg Thr Ala Phe Arg
1765            1770            1775

AAC AAG CTG CCT TTG ACG TTC GGC GAC TTT GAC GAG CAC GAG GTC   5468
Asn Lys Leu Pro Leu Thr Phe Gly Asp Phe Asp Glu His Glu Val
1780            1785            1790

GAT GCG TTG GCC TCC GGG ATT ACT TTC GGA GAC TTC GAC GAC GTC   5513
Asp Ala Leu Ala Ser Gly Ile Thr Phe Gly Asp Phe Asp Asp Val
1795            1800            1805

CTG CGA CTA GGC CGC GCG GGT GCA TAT ATT TTC TCC TCG GAC ACT   5558
Leu Arg Leu Gly Arg Ala Gly Ala Tyr Ile Phe Ser Ser Asp Thr
1810            1815            1820

GGC AGC GGA CAT TTA CAA CAA AAA TCC GTT AGG CAG CAC AAT CTC   5603
Gly Ser Gly His Leu Gln Gln Lys Ser Val Arg Gln His Asn Leu
1825            1830            1835

CAG TGC GCA CAA CTG GAT GCG GTC CAG GAG GAG AAA ATG TAC CCG   5648
Gln Cys Ala Gln Leu Asp Ala Val Gln Glu Glu Lys Met Tyr Pro
1840            1845            1850

CCA AAA TTG GAT ACT GAG AGG GAG AAG CTG TTG CTG CTG AAA ATG   5693
Pro Lys Leu Asp Thr Glu Arg Glu Lys Leu Leu Leu Leu Lys Met
1855            1860            1865
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SUBSTITUTE SHEET

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CAG ATG CAC CCA TCG GAG GCT AAT AAG AGT CGA TAC CAG TCT CGC   5738
Gln Met His Pro Ser Glu Ala Asn Lys Ser Arg Tyr Gln Ser Arg
1870          1875          1880

AAA GTG GAG AAC ATG AAA GCC ACG GTG GTG GAC AGG CTC ACA TCG   5783
Lys Val Glu Asn Met Lys Ala Thr Val Val Asp Arg Leu Thr Ser
1885          1890          1895

GGG GCC AGA TTG TAC ACG GGA GCG GAC GTA GGC CGC ATA CCA ACA   5828
Gly Ala Arg Leu Tyr Thr Gly Ala Asp Val Gly Arg Ile Pro Thr
1900          1905          1910

TAC GCG GTT CGG TAC CCC CGC CCC GTG TAC TCC CCT ACC GTG ATC   5873
Tyr Ala Val Arg Tyr Pro Arg Pro Val Tyr Ser Pro Thr Val Ile
1915          1920          1925

GAA AGA TTC TCA AGC CCC GAT GTA GCA ATC GCA GCG TGC AAC GAA   5918
Glu Arg Phe Ser Ser Pro Asp Val Ala Ile Ala Ala Cys Asn Glu
1930          1935          1940

TAC CTA TCC AGA AAT TAC CCA ACA GTG GCG TCG TAC CAG ATA ACA   5963
Tyr Leu Ser Arg Asn Tyr Pro Thr Val Ala Ser Tyr Gln Ile Thr
1945          1950          1955

GAT GAA TAC GAC GCA TAC TTG GAC ATG GTT GAC GGG TCG GAT AGT   6008
Asp Glu Tyr Asp Ala Tyr Leu Asp Met Val Asp Gly Ser Asp Ser
1960          1965          1970

TGC TTG GAC AGA GCG ACA TTC TGC CCG GCG AAG CTC CGG TGC TAC   6053
Cys Leu Asp Arg Ala Thr Phe Cys Pro Ala Lys Leu Arg Cys Tyr
1975          1980          1985

CCG AAA CAT CAT GCG TAC CAC CAG CCG ACT GTA CGC AGT GCC GTC   6098
Pro Lys His His Ala Tyr His Gln Pro Thr Val Arg Ser Ala Val
1990          1995          2000

CCG TCA CCC TTT CAG AAC ACA CTA CAG AAC GTG CTA GCG GCC GCC   6143
Pro Ser Pro Phe Gln Asn Thr Leu Gln Asn Val Leu Ala Ala Ala
2005          2010          2015

ACC AAG AGA AAC TGC AAC GTC ACG CAA ATG CGA GAA CTA CCC ACC   6188
Thr Lys Arg Asn Cys Asn Val Thr Gln Met Arg Glu Leu Pro Thr
2020          2025          2030

ATG GAC TCG GCA GTG TTC AAC GTG GAG TGC TTC AAG CGC TAT GCC   6233
Met Asp Ser Ala Val Phe Asn Val Glu Cys Phe Lys Arg Tyr Ala
2035          2040          2045

TGC TCC GGA GAA TAT TGG GAA GAA TAT GCT AAA CAA CCT ATC CGG   6278
Cys Ser Gly Glu Tyr Trp Glu Glu Tyr Ala Lys Gln Pro Ile Arg
2050          2055          2060

ATA ACC ACT GAG AAC ATC ACT ACC TAT GTG ACC AAA TTG AAA GGC   6323
Ile Thr Thr Glu Asn Ile Thr Thr Tyr Val Thr Lys Leu Lys Gly
2065          2070          2075
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SUBSTITUTE SHEET

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CCG AAA GCT GCT GCC TTG TTC GCT AAG ACC CAC AAC TTG GTT CCG   6368
Pro Lys Ala Ala Ala Leu Phe Ala Lys Thr His Asn Leu Val Pro
2080          2085              2090


CTG CAG GAG GTT CCC ATG GAC AGA TTC ACG GTC GAC ATG AAA CGA   6413
Leu Gln Glu Val Pro Met Asp Arg Phe Thr Val Asp Met Lys Arg
2095          2100              2105


GAT GTC AAA GTC ACT CCA GGG ACG AAA CAC ACA GAG GAA AGA CCC   6458
Asp Val Lys Val Thr Pro Gly Thr Lys His Thr Glu Glu Arg Pro
2110          2115              2120


AAA GTC CAG GTA ATT CAA GCA GCG GAG CCA TTG GCG ACC GCT TAC   6503
Lys Val Gln Val Ile Gln Ala Ala Glu Pro Leu Ala Thr Ala Tyr
2125          2130              2135


CTG TGC GGC ATC CAC AGG GAA TTA GTA AGG AGA CTA AAT GCT GTG   6548
Leu Cys Gly Ile His Arg Glu Leu Val Arg Arg Leu Asn Ala Val
2140          2145              2150


TTA CGC CCT AAC GTG CAC ACA TTG TTT GAT ATG TCG GCC GAA GAC   6593
Leu Arg Pro Asn Val His Thr Leu Phe Asp Met Ser Ala Glu Asp
2155          2160              2165


TTT GAC GCG ATC ATC GCC TCT CAC TTC CAC CCA GGA GAC CCG GTT   6638
Phe Asp Ala Ile Ile Ala Ser His Phe His Pro Gly Asp Pro Val
2170          2175              2180


CTA GAG ACG GAC ATT GCA TCA TTC GAC AAA AGC CAG GAC GAC TCC   6683
Leu Glu Thr Asp Ile Ala Ser Phe Asp Lys Ser Gln Asp Asp Ser
2185          2190              2195


TTG GCT CTT ACA GGT TTA ATG ATC CTC GAA GAT CTA GGG GTG GAT   6728
Leu Ala Leu Thr Gly Leu Met Ile Leu Glu Asp Leu Gly Val Asp
2200          2205              2210


CAG TAC CTG CTG GAC TTG ATC GAG GCA GCC TTT GGG GAA ATA TCC   6773
Gln Tyr Leu Leu Asp Leu Ile Glu Ala Ala Phe Gly Glu Ile Ser
2215          2220              2225


AGC TGT CAC CTA CCA ACT GGC ACG CGC TTC AAG TTC GGA GCT ATG   6818
Ser Cys His Leu Pro Thr Gly Thr Arg Phe Lys Phe Gly Ala Met
2230          2235              2240


ATG AAA TCG GGC ATG TTT CTG ACT TTG TTT ATT AAC ACT GTT TTG   6863
Met Lys Ser Gly Met Phe Leu Thr Leu Phe Ile Asn Thr Val Leu
2245          2250              2255


AAC ATC ACC ATA GCA AGC AGG GTA CTG GAG CAG AGA CTC ACT GAC   6908
Asn Ile Thr Ile Ala Ser Arg Val Leu Glu Gln Arg Leu Thr Asp
2260          2265              2270


TCC GCC TGT GCG GCC TTC ATC GGC GAC GAC AAC ATC GTT CAC GGA   6953
Ser Ala Cys Ala Ala Phe Ile Gly Asp Asp Asn Ile Val His Gly
2275          2280              2285
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SUBSTITUTE SHEET

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GTG ATC TCC GAC AAG CTG ATG GCG GAG AGG TGC GCG TCG TGG GTC  6998
Val Ile Ser Asp Lys Leu Met Ala Glu Arg Cys Ala Ser Trp Val
2290          2295          2300

AAC ATG GAG GTG AAG ATC ATT GAC GCT GTC ATG GGC GAA AAA CCC  7043
Asn Met Glu Val Lys Ile Ile Asp Ala Val Met Gly Glu Lys Pro
2305          2310          2315

CCA TAT TTT TGT GGG GGA TTC ATA GTT TTT GAC AGC GTC ACA CAG  7088
Pro Tyr Phe Cys Gly Gly Phe Ile Val Phe Asp Ser Val Thr Gln
2320          2325          2330

ACC GCC TGC CGT GTT TCA GAC CCA CTT AAG CGC CTG TTC AAG TTG  7133
Thr Ala Cys Arg Val Ser Asp Pro Leu Lys Arg Leu Phe Lys Leu
2335          2340          2345

GGT AAG CCG CTA ACA GCT GAA GAC AAG CAG GAC GAA GAC AGG CGA  7178
Gly Lys Pro Leu Thr Ala Glu Asp Lys Gln Asp Glu Asp Arg Arg
2350          2355          2360

CGA GCA CTG AGT GAC GAG GTT AGC AAG TGG TTC CGG ACA GGC TTG  7223
Arg Ala Leu Ser Asp Glu Val Ser Lys Trp Phe Arg Thr Gly Leu
2365          2370          2375

GGG GCC GAA CTG GAG GTG GCA CTA ACA TCT AGG TAT GAG GTA GAG  7268
Gly Ala Glu Leu Glu Val Ala Leu Thr Ser Arg Tyr Glu Val Glu
2380          2385          2390

GGC TGC AAA AGT ATC CTC ATA GCC ATG ACC ACC TTG GCG AGG GAC  7313
Gly Cys Lys Ser Ile Leu Ile Ala Met Thr Thr Leu Ala Arg Asp
2395          2400          2405

ATT AAG GCG TTT AAG AAA TTG AGA GGA CCT GTT ATA CAC CTC TAC  7358
Ile Lys Ala Phe Lys Lys Leu Arg Gly Pro Val Ile His Leu Tyr
2410          2415          2420

GGC GGT CCT AGA TTG GTG CGT TAA TACACAGAAT TCTGATTATA GCGCACTATT  7412
Gly Gly Pro Arg Leu Val Arg
2425          2430

ATAGCACC ATG AAT TAC ATC CCT ACG CAA ACG TTT TAC GGC CGC CGG  7459
         Met Asn Tyr Ile Pro Thr Gln Thr Phe Tyr Gly Arg Arg
              5                   10

TGG CGC CCG CGC CCG GCG GCC CGT CCT TGG CCG TTG CAG GCC ACT  7504
Trp Arg Pro Arg Pro Ala Ala Arg Pro Trp Pro Leu Gln Ala Thr
    15                  20                  25

CCG GTG GCT CCC GTC GTC CCC GAC TTC CAG GCC CAG CAG ATG CAG  7549
Pro Val Ala Pro Val Val Pro Asp Phe Gln Ala Gln Gln Met Gln
    30                  35                  40

CAA CTC ATC AGC GCC GTA AAT GCG CTG ACA ATG AGA CAG AAC GCA  7594
Gln Leu Ile Ser Ala Val Asn Ala Leu Thr Met Arg Gln Asn Ala
    45                  50                  55
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SUBSTITUTE SHEET

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ATT GCT CCT GCT AGG CCT CCC AAA CCA AAG AAG AAG AAG ACA ACC   7639
Ile Ala Pro Ala Arg Pro Pro Lys Pro Lys Lys Lys Lys Thr Thr
    60                  65                  70

AAA CCA AAG CCG AAA ACG CAG CCC AAG AAG ATC AAC GGA AAA ACG   7684
Lys Pro Lys Pro Lys Thr Gln Pro Lys Lys Ile Asn Gly Lys Thr
    75                  80                  85

CAG CAG CAA AAG AAG AAA GAC AAG CAA GCC GAC AAG AAG AAG AAG   7729
Gln Gln Gln Lys Lys Lys Asp Lys Gln Ala Asp Lys Lys Lys Lys
    90                  95                  100

AAA CCC GGA AAA AGA GAA AGA ATG TGC ATG AAG ATT GAA AAT GAC   7774
Lys Pro Gly Lys Arg Glu Arg Met Cys Met Lys Ile Glu Asn Asp
    105                 110                 115

TGT ATC TTC GAA GTC AAA CAC GAA GGA AAG GTC ACT GGG TAC GCC   7819
Cys Ile Phe Glu Val Lys His Glu Gly Lys Val Thr Gly Tyr Ala
    120                 125                 130

TGC CTG GTG GGC GAC AAA GTC ATG AAA CCT GCC CAC GTG AAA GGA   7864
Cys Leu Val Gly Asp Lys Val Met Lys Pro Ala His Val Lys Gly
    135                 140                 145

GTC ATC GAC AAC GCG GAC CTG GCA AAG CTA GCT TTC AAG AAA TCG   7909
Val Ile Asp Asn Ala Asp Leu Ala Lys Leu Ala Phe Lys Lys Ser
    150                 155                 160

AGC AAG TAT GAC CTT GAG TGT GCC CAG ATA CCA GTT CAC ATG AGG   7954
Ser Lys Tyr Asp Leu Glu Cys Ala Gln Ile Pro Val His Met Arg
    165                 170                 175

TCG GAT GCC TCA AAG TAC ACG CAT GAG AAG CCC GAG GGA CAC TAT   7999
Ser Asp Ala Ser Lys Tyr Thr His Glu Lys Pro Glu Gly His Tyr
    180                 185                 190

AAC TGG CAC CAC GGG GCT GTT CAG TAC AGC GGA GGT AGG TTC ACT   8044
Asn Trp His His Gly Ala Val Gln Tyr Ser Gly Gly Arg Phe Thr
    195                 200                 205

ATA CCG ACA GGA GCG GGC AAA CCG GGA GAC AGT GGC CGG CCC ATC   8089
Ile Pro Thr Gly Ala Gly Lys Pro Gly Asp Ser Gly Arg Pro Ile
    210                 215                 220

TTT GAC AAC AAG GGG AGG GTA GTC GCT ATC GTC CTG GGC GGG GCC   8134
Phe Asp Asn Lys Gly Arg Val Val Ala Ile Val Leu Gly Gly Ala
    225                 230                 235

AAC GAG GGC TCA CGC ACA GCA CTG TCG GTG GTC ACC TGG AAC AAA   8179
Asn Glu Gly Ser Arg Thr Ala Leu Ser Val Val Thr Trp Asn Lys
    240                 245                 250

GAT ATG GTG ACT AGA GTG ACC CCC GAG GGG TCC GAA GAG TGG TCC   8224
Asp Met Val Thr Arg Val Thr Pro Glu Gly Ser Glu Glu Trp Ser
    255                 260                 265
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GCC CCG CTG ATT ACT GCC ATG TGT GTC CTT GCC AAT GCT ACC TTC   8269
Ala Pro Leu Ile Thr Ala Met Cys Val Leu Ala Asn Ala Thr Phe
    270   275   280

CCG TGC TTC CAG CCC CCG TGT GTA CCT TGC TGC TAT GAA AAC AAC   8314
Pro Cys Phe Gln Pro Pro Cys Val Pro Cys Cys Tyr Glu Asn Asn
    285   290   295

GCA GAG GCC ACA CTA CGG ATG CTC GAG GAT AAC GTG GAT AGG CCA   8359
Ala Glu Ala Thr Leu Arg Met Leu Glu Asp Asn Val Asp Arg Pro
    300   305   310

GGG TAC TAC GAC CTC CTT CAG GCA GCC TTG ACG TGC CGA AAC GGA   8404
Gly Tyr Tyr Asp Leu Leu Gln Ala Ala Leu Thr Cys Arg Asn Gly
    315   320   325

ACA AGA CAC CGG CGC AGC GTG TCG CAA CAC TTC AAC GTG TAT AAG   8449
Thr Arg His Arg Arg Ser Val Ser Gln His Phe Asn Val Tyr Lys
    330   335   340

GCT ACA CGC CCT TAC ATC GCG TAC TGC GCC GAC TGC GGA GCA GGG   8494
Ala Thr Arg Pro Tyr Ile Ala Tyr Cys Ala Asp Cys Gly Ala Gly
    345   350   355

CAC TCG TGT CAT AGC CCC GTA GCA ATT GAA GCG GTC AGG TCC GAA   8539
His Ser Cys His Ser Pro Val Ala Ile Glu Ala Val Arg Ser Glu
    360   365   370

GCT ACC GAC GGG ATG CTG AAG ATT CAG TTC TCG GCA CAA ATT GGC   8584
Ala Thr Asp Gly Met Leu Lys Ile Gln Phe Ser Ala Gln Ile Gly
    375   380   385

ATA GAT AAG AGT GAC AAT CAT GAC TAC ACG AAG ATA AGG TAC GCA   8629
Ile Asp Lys Ser Asp Asn His Asp Tyr Thr Lys Ile Arg Tyr Ala
    390   395   400

GAC GGG CAC GCC ATT GAG AAT GCC GTC CGG TCA TCT TTG AAG GTA   8674
Asp Gly His Ala Ile Glu Asn Ala Val Arg Ser Ser Leu Lys Val
    405   410   415

GCC ACC TCC GGA GAC TGT TTC GTC CAT GGC ACA ATG GGA CAT TTC   8719
Ala Thr Ser Gly Asp Cys Phe Val His Gly Thr Met Gly His Phe
    420   425   430

ATA CTG GCA AAG TGC CCA CCG GGT GAA TTC CTG CAG GTC TCG ATC   8764
Ile Leu Ala Lys Cys Pro Pro Gly Glu Phe Leu Gln Val Ser Ile
    435   440   445

CAG GAC ACC AGA AAC GCG GTC CGT GCC TGC AGA ATA CAA TAT CAT   8809
Gln Asp Thr Arg Asn Ala Val Arg Ala Cys Arg Ile Gln Tyr His
    450   455   460

CAT GAC CCT CAA CCG GTG GGT AGA GAA AAA TTT ACA ATT AGA CCA   8854
His Asp Pro Gln Pro Val Gly Arg Glu Lys Phe Thr Ile Arg Pro
    465   470   475

CAC TAT GGA AAA GAG ATC CCT TGC ACC ACT TAT CAA CAG ACC ACA   8899
His Tyr Gly Lys Glu Ile Pro Cys Thr Thr Tyr Gln Gln Thr Thr
    480   485   490

GCG AAG ACC GTG GAG GAA ATC GAC ATG CAT ATG CCG CCA GAT ACG   8944
Ala Lys Thr Val Glu Glu Ile Asp Met His Met Pro Pro Asp Thr
    495   500   505

CCG GAC AGG ACG TTG CTA TCA CAG CAA TCT GGC AAT GTA AAG ATC   8989
Pro Asp Arg Thr Leu Leu Ser Gln Gln Ser Gly Asn Val Lys Ile
    510   515   520

ACA GTC GGA GGA AAG AAG GTG AAA TAC AAC TGC ACC TGT GGA ACC   9034
Thr Val Gly Gly Lys Lys Val Lys Tyr Asn Cys Thr Cys Gly Thr
    525   530   535

GGA AAC GTT GGC ACT ACT AAT TCG GAC ATG ACG ATC AAC ACG TGT   9079
Gly Asn Val Gly Thr Thr Asn Ser Asp Met Thr Ile Asn Thr Cys
    540   545   550

CTA ATA GAG CAG TGC CAC GTC TCA GTG ACG GAC CAT AAG AAA TGG   9124
Leu Ile Glu Gln Cys His Val Ser Val Thr Asp His Lys Lys Trp
    555   560   565

CAG TTC AAC TCA CCT TTC GTC CCG AGA GCC GAC GAA CCG GCT AGA   9169
Gln Phe Asn Ser Pro Phe Val Pro Arg Ala Asp Glu Pro Ala Arg
    570   575   580

AAA GGC AAA GTC CAT ATC CCA TTC CCG TTG GAC AAC ATC ACA TGC   9214
Lys Gly Lys Val His Ile Pro Phe Pro Leu Asp Asn Ile Thr Cys
    585   590   595

AGA GTT CCA ATG GCG CGC GAA CCA ACC GTC ATC CAC GGC AAA AGA   9259
Arg Val Pro Met Ala Arg Glu Pro Thr Val Ile His Gly Lys Arg
    600   605   610

GAA GTG ACA CTG CAC CTT CAC CCA GAT CAT CCC ACG CTC TTT TCC   9304
Glu Val Thr Leu His Leu His Pro Asp His Pro Thr Leu Phe Ser
    615   620   625

TAC CGC ACA CTG GGT GAG GAC CCG CAG TAT CAC GAG GAA TGG GTG   9349
Tyr Arg Thr Leu Gly Glu Asp Pro Gln Tyr His Glu Glu Trp Val
    630   635   640

ACA GCG GCG GTG GAA CGG ACC ATA CCC GTA CCA GTG GAC GGG ATG   9394
Thr Ala Ala Val Glu Arg Thr Ile Pro Val Pro Val Asp Gly Met
    645   650   655

GAG TAC CAC TGG GGA AAC AAC GAC CCA GTG AGG CTT TGG TCT CAA   9439
Glu Tyr His Trp Gly Asn Asn Asp Pro Val Arg Leu Trp Ser Gln
    660   665   670

CTC ACC ACT GAA GGG AAA CCG CAC GGC TGG CCG CAT CAG ATC GTA   9484
Leu Thr Thr Glu Gly Lys Pro His Gly Trp Pro His Gln Ile Val
    675   680   685

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CAG TAC TAC TAT GGG CTT TAC CCG GCC GCT ACA GTA TCC GCG GTC   9529
Gln Tyr Tyr Tyr Gly Leu Tyr Pro Ala Ala Thr Val Ser Ala Val
    690             695             700

GTC GGG ATG AGC TTA CTG GCG TTG ATA TCG ATC TTC GCG TCG TGC   9574
Val Gly Met Ser Leu Leu Ala Leu Ile Ser Ile Phe Ala Ser Cys
    705             710             715

TAC ATG CTG GTT GCG GCC CGC AGT AAG TGC TTG ACC CCT TAT GCT   9619
Tyr Met Leu Val Ala Ala Arg Ser Lys Cys Leu Thr Pro Tyr Ala
    720             725             730

TTA ACA CCA GGA GCT GCA GTT CCG TGG ACG CTG GGG ATA CTC TGC   9664
Leu Thr Pro Gly Ala Ala Val Pro Trp Thr Leu Gly Ile Leu Cys
    735             740             745

TGC GCC CCG CGG GCG CAC GCA GCT AGT GTG GCA GAG ACT ATG GCC   9709
Cys Ala Pro Arg Ala His Ala Ala Ser Val Ala Glu Thr Met Ala
    750             755             760

TAC TTG TGG GAC CAA AAC CAA GCG TTG TTC TGG TTG GAG TTT GCG   9754
Tyr Leu Trp Asp Gln Asn Gln Ala Leu Phe Trp Leu Glu Phe Ala
    765             770             775

GCC CCT GTT GCC TGC ATC CTC ATC ATC ACG TAT TGC CTC AGA AAC   9799
Ala Pro Val Ala Cys Ile Leu Ile Ile Thr Tyr Cys Leu Arg Asn
    780             785             790

GTG CTG TGT TGC TGT AAG AGC CTT TCT TTT TTA GTG CTA CTG AGC   9844
Val Leu Cys Cys Cys Lys Ser Leu Ser Phe Leu Val Leu Leu Ser
    795             800             805

CTC GGG GCA ACC GCC AGA GCT TAC GAA CAT TCG ACA GTA ATG CCG   9889
Leu Gly Ala Thr Ala Arg Ala Tyr Glu His Ser Thr Val Met Pro
    810             815             820

AAC GTG GTG GGG TTC CCG TAT AAG GCT CAC ATT GAA AGG CCA GGA   9934
Asn Val Val Gly Phe Pro Tyr Lys Ala His Ile Glu Arg Pro Gly
    825             830             835

TAT AGC CCC CTC ACT TTG CAG ATG CAG GTT GTT GAA ACC AGC CTC   9979
Tyr Ser Pro Leu Thr Leu Gln Met Gln Val Val Glu Thr Ser Leu
    840             845             850

GAA CCA ACC CTT AAT TTG GAA TAC ATA ACC TGT GAG TAC AAG ACG   10024
Glu Pro Thr Leu Asn Leu Glu Tyr Ile Thr Cys Glu Tyr Lys Thr
    855             860             865

GTC GTC CCG TCG CCG TAC GTG AAG TGC TGC GGC GCC TCA GAG TGC   10069
Val Val Pro Ser Pro Tyr Val Lys Cys Cys Gly Ala Ser Glu Cys
    870             875             880

TCC ACT AAA GAG AAG CCT GAC TAC CAA TGC AAG GTT TAC ACA GGC   10114
Ser Thr Lys Glu Lys Pro Asp Tyr Gln Cys Lys Val Tyr Thr Gly
    885             890             895
```

```
GTG TAC CCG TTC ATG TGG GGA GGG GCA TAT TGC TTC TGC GAC TCA   10159
Val Tyr Pro Phe Met Trp Gly Gly Ala Tyr Cys Phe Cys Asp Ser
    900             905             910

GAA AAC ACG CAA CTC AGC GAG GCG TAC GTC GAT CGA TCG GAC GTA   10204
Glu Asn Thr Gln Leu Ser Glu Ala Tyr Val Asp Arg Ser Asp Val
    915             920             925

TGC AGG CAT GAT CAC GCA TCT GCT TAC AAA GCC CAT ACA GCA TCG   10249
Cys Arg His Asp His Ala Ser Ala Tyr Lys Ala His Thr Ala Ser
    930             935             940

CTG AAG GCC AAA GTG AGG GTT ATG TAC GGC AAC GTA AAC CAG ACT   10294
Leu Lys Ala Lys Val Arg Val Met Tyr Gly Asn Val Asn Gln Thr
    945             950             955

GTG GAT GTT TAC GTG AAC GGA GAC CAT GCC GTC ACG ATA GGG GGT   10339
Val Asp Val Tyr Val Asn Gly Asp His Ala Val Thr Ile Gly Gly
    960             965             970

ACT CAG TTC ATA TTC GGG CCG CTG TCA TCG GCC TGG ACC CCG TTC   10384
Thr Gln Phe Ile Phe Gly Pro Leu Ser Ser Ala Trp Thr Pro Phe
    975             980             985

GAC AAC AAG ATA GTC GTG TAC AAA GAC GAA GTG TTC AAT CAG GAC   10429
Asp Asn Lys Ile Val Val Tyr Lys Asp Glu Val Phe Asn Gln Asp
    990             995             1000

TTC CCG CCG TAC GGA TCT GGG CAA CCA GGG CGC TTC GGC GAC ATC   10474
Phe Pro Pro Tyr Gly Ser Gly Gln Pro Gly Arg Phe Gly Asp Ile
    1005            1010            1015

CAA AGC AGA ACA GTG GAG AGT AAC GAC CTG TAC GCG AAC ACG GCA   10519
Gln Ser Arg Thr Val Glu Ser Asn Asp Leu Tyr Ala Asn Thr Ala
    1020            1025            1030

CTG AAG CTG GCA CGC CCT TCA CCC GGC ATG GTC CAT GTA CCG TAC   10564
Leu Lys Leu Ala Arg Pro Ser Pro Gly Met Val His Val Pro Tyr
    1035            1040            1045

ACA CAG ACA CCT TCA GGG TTC AAA TAT TGG CTA AAG GAA AAA GGG   10609
Thr Gln Thr Pro Ser Gly Phe Lys Tyr Trp Leu Lys Glu Lys Gly
    1050            1055            1060

ACA GCC CTA AAT ACG AAG GCT CCT TTT GGC TGC CAA ATC AAA ACG   10654
Thr Ala Leu Asn Thr Lys Ala Pro Phe Gly Cys Gln Ile Lys Thr
    1065            1070            1075

AAC CCT GTC AGG GCC ATG AAC TGC GCC GTG GGA AAC ATC CCT GTC   10699
Asn Pro Val Arg Ala Met Asn Cys Ala Val Gly Asn Ile Pro Val
    1080            1085            1090

TCC ATG AAT TTG CCT GAC AGC GCC TTT ACC CGC ATT GTC GAG GCG   10744
Ser Met Asn Leu Pro Asp Ser Ala Phe Thr Arg Ile Val Glu Ala
    1095            1100            1105
```

CCG ACC ATC ATT GAC CTG ACT TGC ACA GTG GCT ACC TGT ACG CAC   10789
Pro Thr Ile Ile Asp Leu Thr Cys Thr Val Ala Thr Cys Thr His
1110   1115   1120

TCC TCG GAT TTC GGC GGC GTC TTG ACA CTG ACG TAC AAG ACC AAC   10834
Ser Ser Asp Phe Gly Gly Val Leu Thr Leu Thr Tyr Lys Thr Asn
1125   1130   1135

AAG AAC GGG GAC TGC TCT GTA CAC TCG CAC TCT AAC GTA GCT ACT   10879
Lys Asn Gly Asp Cys Ser Val His Ser His Ser Asn Val Ala Thr
1140   1145   1150

CTA CAG GAG GCC ACA GCA AAA GTG AAG ACA GCA GGT AAG GTG ACC   10924
Leu Gln Glu Ala Thr Ala Lys Val Lys Thr Ala Gly Lys Val Thr
1155   1160   1165

TTA CAC TTC TCC ACG GCA AGC GCA TCA CCT TCT TTT GTG GTG TCG   10969
Leu His Phe Ser Thr Ala Ser Ala Ser Pro Ser Phe Val Val Ser
1170   1175   1180

CTA TGC AGT GCT AGG GCC ACC TGT TCA GCG TCG TGT GAG CCC CCG   11014
Leu Cys Ser Ala Arg Ala Thr Cys Ser Ala Ser Cys Glu Pro Pro
1185   1190   1195

AAA GAC CAC ATA GTC CCA TAT GCG GCT AGC CAC AGT AAC GTA GTG   11059
Lys Asp His Ile Val Pro Tyr Ala Ala Ser His Ser Asn Val Val
1200   1205   1210

TTT CCA GAC ATG TCG GGC ACC GCA CTA TCA TGG GTG CAG AAA ATC   11104
Phe Pro Asp Met Ser Gly Thr Ala Leu Ser Trp Val Gln Lys Ile
1215   1220   1225

TCG GGT GGT CTG GGG GCC TTC GCA ATC GGC GCT ATC CTG GTG CTG   11149
Ser Gly Gly Leu Gly Ala Phe Ala Ile Gly Ala Ile Leu Val Leu
1230   1235   1240

GTT GTG GTC ACT TGC ATT GGG CTC CGC AGA TAA GTTAGGGTAG   11192
Val Val Val Thr Cys Ile Gly Leu Arg Arg
1245   1250

GCAATGGCAT TGATATAGCA AGAAAATTGA AAACAGAAAA AGTTAGGGTA AGCAATGGCA   11252

TATAACCATA ACTGTATAAC TTGTAACAAA GCGCAACAAG ACCTGCGCAA TTGGCCCCGT   11312

GGTCCGCCTC ACGGAAACTC GGGGCAACTC ATATTGACAC ATTAATTGGC AATAATTGGA   11372

AGCTTACATA AGCTTAATTC GACGAATAAT TGGATTTTTA TTTTATTTTG CAATTGGTTT   11432

TTAATATTTC CAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA   11492

AAAAAAAAAA AAAAAAAAAA ACTAG   11517

SUBSTITUTE SHEET

Figure 6

Figure 7 (1)

Figure 7 (2)

Figure 7 (3)

Figure 7 layout scheme

Figure 7 (1)

HindIII-SphI-PstI-SalI-XbaI-BamHI-SmaI-KpnI-SacI-EcoRI

XhoI 458

EcoRI 1405

M13mp19-SFV.RI
8605 bp

SFV

DAccI (308) - AccI (6399)

F

SacI 1801
HindIII 1947
EcoRI 2186
KpnI 3597
EcoRI 3764
KpnI 5035
XhoI 5306
Kpn1 5841
XbaI 6715
BglII 6715
EcoRI 7391

SphI 13975
XmnI 13262
SpeI 11515
HindIII 11384
HindIII 11374
NdeI 11033

NdeI 8929
NsiI 8927
EcoRI 8746
XhoI 8338
AsuII 7783

pSP6-SFV4
14411 bp

SP6   nsP1
Ap
ori
E1
6K
E2
E3   C
nsp4
nsP3
nsP2

(SmaI-XmaI-BamHI)

E | D AsuII-NdeI

Figure 7 (2)

Figure 7 (3)

Figure 8

Figure 9
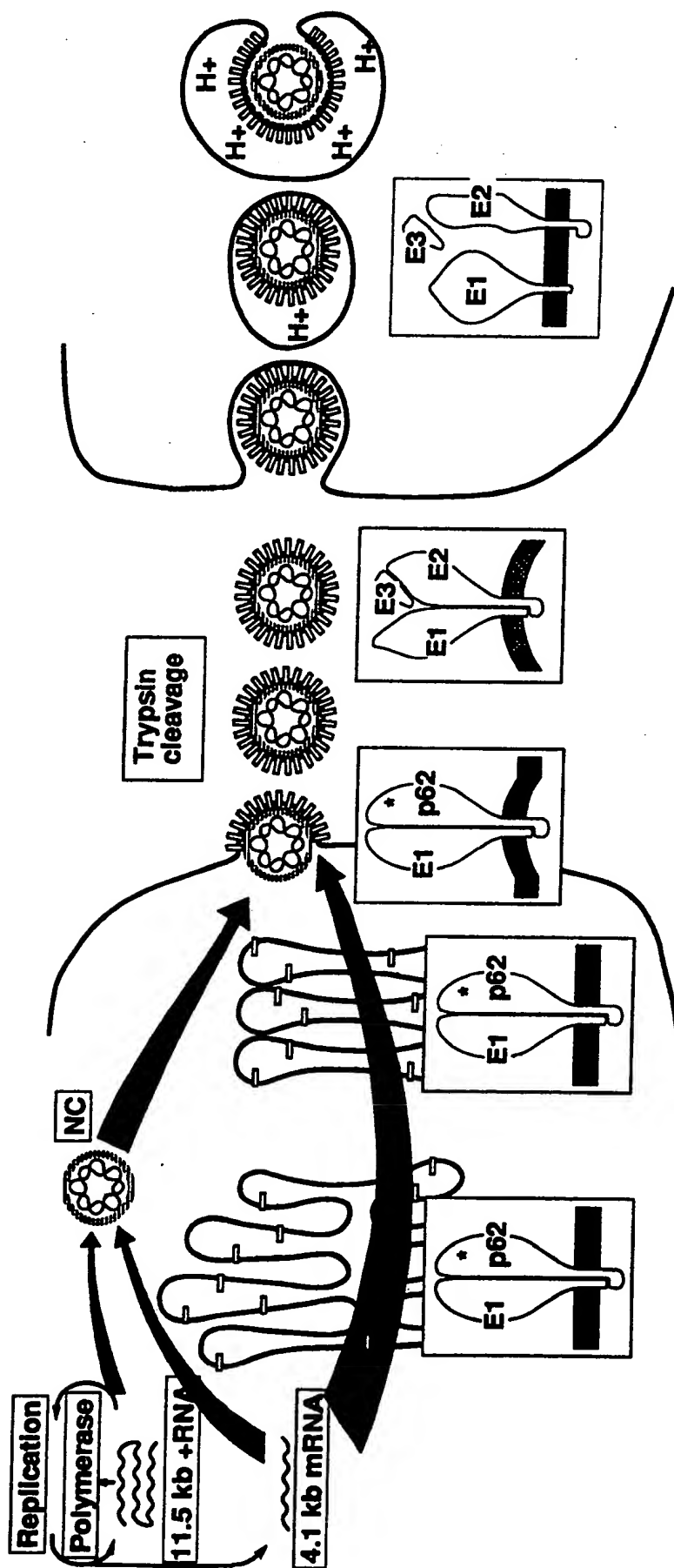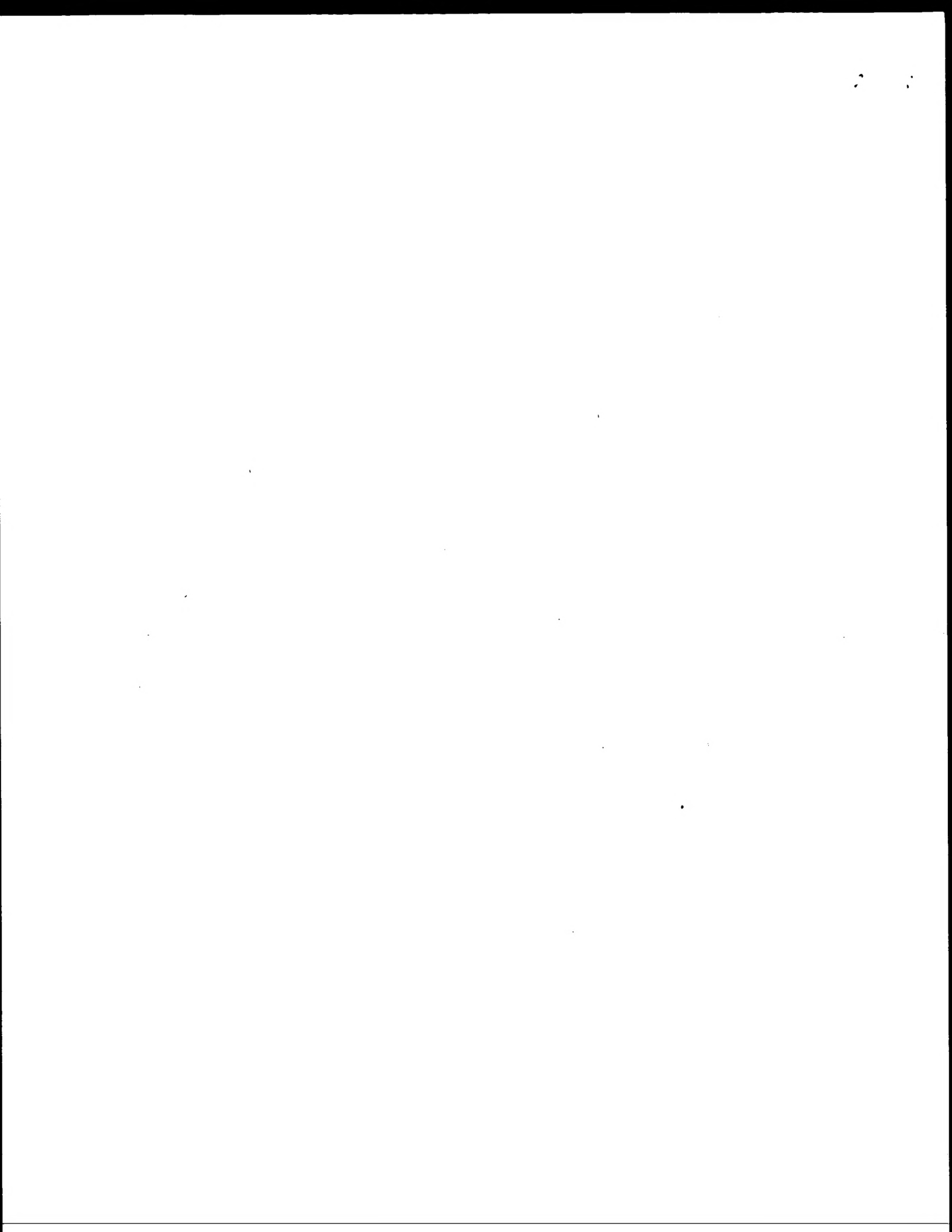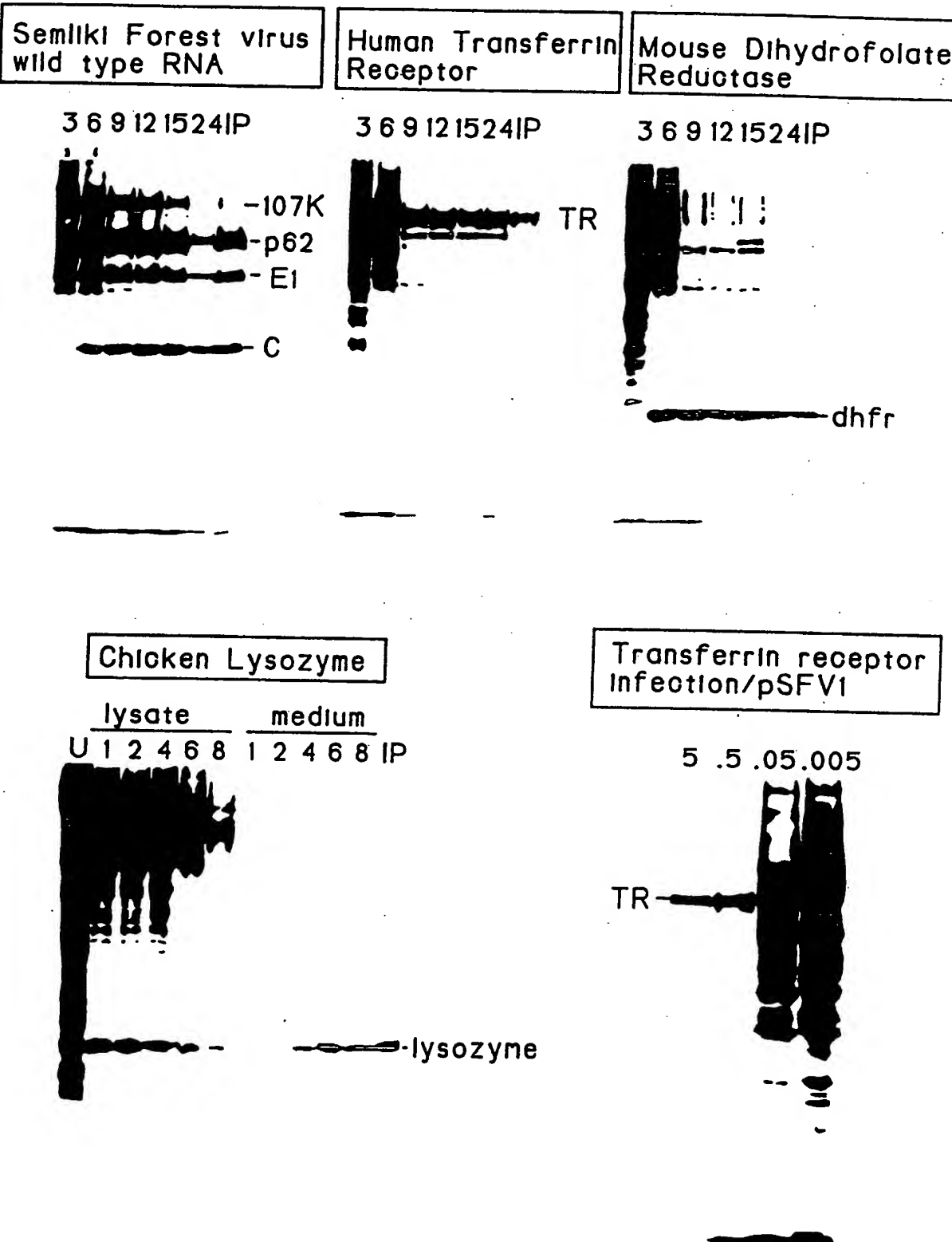
Figure 10

Figure 11

```
                              BamH1
--- --- --- --- -G GAT CC- --- --- ---          SFV vector E2
AAC TCA CCT TTC GTC CCG AGA GCC GAC GAA CCG GCT AGA AAA GGC AAA GTC CAT   SFV E2
Asn Ser Pro Phe Val Pro Arg Ala Asp Glu Pro Ala Arg Lys Gly Lys Val His   SFV E2
--- --- --- --- --- --- --- Glu Asp --- --- --- --- --- --- ---          SFV vector E2
                            246 247
```

```
GAT CCG CGT ATC CAG AGA GGA CCA GGA AGA GCA TTT GTT GAG           HIV·gp120
   GC GCA TAG GTC TCT CCT GGT CCT CGT AAA CAA CTC CTA G           HIV epitope
Asp Pro Arg Ile Gln Arg Gly Pro Gly Arg Ala Phe Val Glu Asp
```

Cut with BamH1
Insert HIV oligo

⟶

```
GAG GAT CCG CGT ATC CAG AGA GGA CCA GGA AGA GCA TTT GTT GAG GAT CCG      SFV-HIV chimera
CTC CTA GGC GCA TAG GTC TCT CCT GGT CCT CGT AAA CAA CTC CTA GGC
Glu Asp Pro Arg Ile Gln Arg Gly Pro Gly Arg Ala Phe Val Glu Asp Pro
                                313
```

Figure 12 (1)
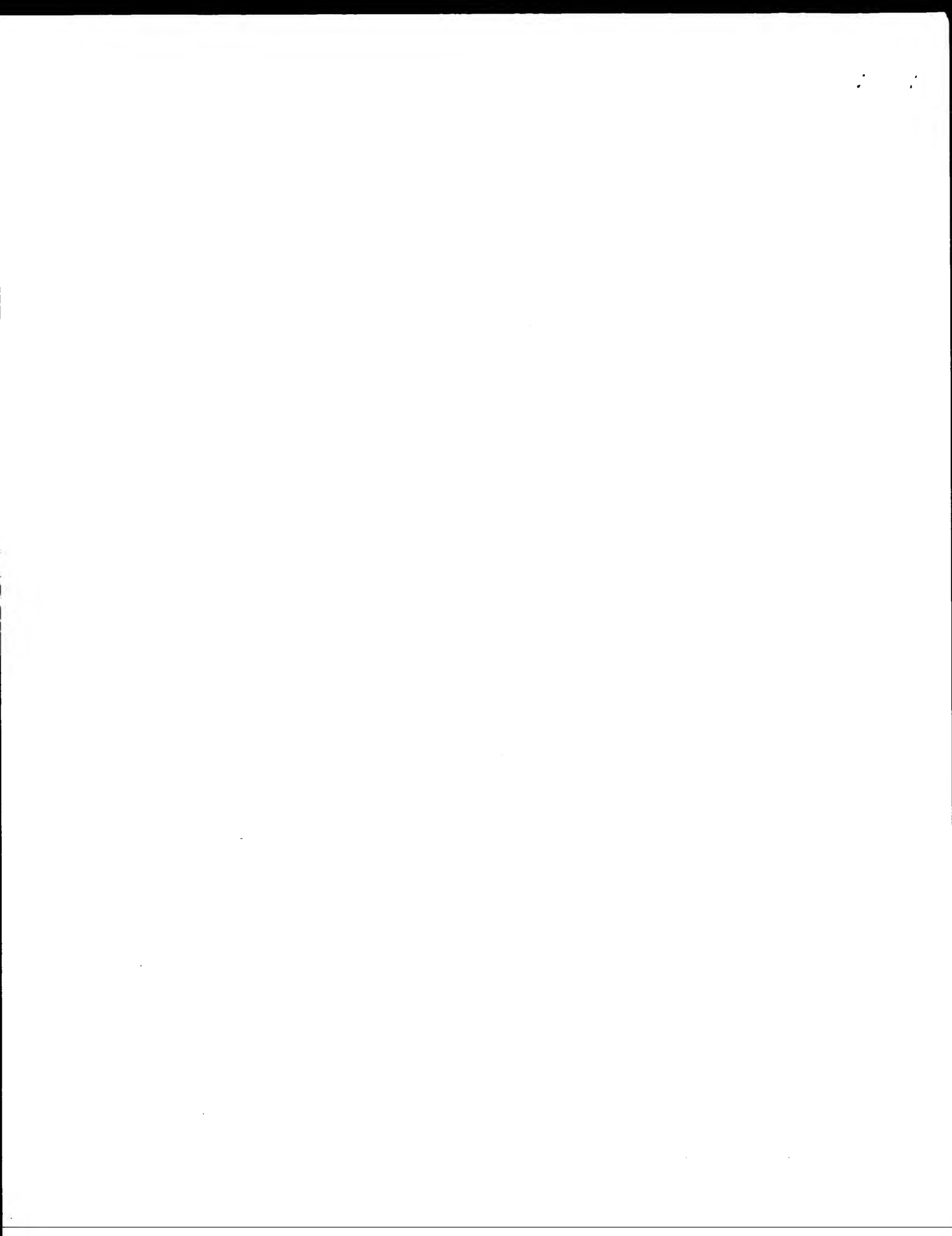
Figure 12 (2)

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 15/86, C 12 N 7/01, A 61 K 39/12

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
IPC5	C 12 N; A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in Fields Searched⁸

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category*	Citation of Document,¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No.¹³
X	PROC.NATL.ACAD.SCI., Vol. 84, 1987 (USA) Robin Levis et al: "Engineered defective interfering RNAs of Sindbis virus express bacterial chloramphenicol acetyltransferase in avian cells", see page 4811 - page 4815 especially page 4811, column 1 lines 1-4,12-15,46-48; column 2 lines 25-28 and page 4812 column 1 lines 24-26 column 2 lines 14-16	1-2,5,9-11,20-22,29,30
Y		3,4,6-8,12-19,23-28,31-41
	--	
Y	WO, A1, 8912095 (APPLIED BIOTECHNOLOGY, INC.) 14 December 1989, see the whole document	3,4,6-8,12-19,23-28,31-41
	--	

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17th March 1992	1992 -03- 19

International Searching Authority	Signature of Authorized Officer
SWEDISH PATENT OFFICE	Carolina Palmcrantz Carolina Palmcrantz

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Dialog Information Services, file 154, MEDLINE 85-91/July, Dialog accession no. 06757425, Medline accession no. 89059425, "Alphaviruses a new vector expressing heterologous genes", Vopr Virusol Jul-Aug 1988, 33 (4) p 502-4	1-2

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 91/00855

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 8912095	89-12-14	AU-D-	3775789	90-01-05